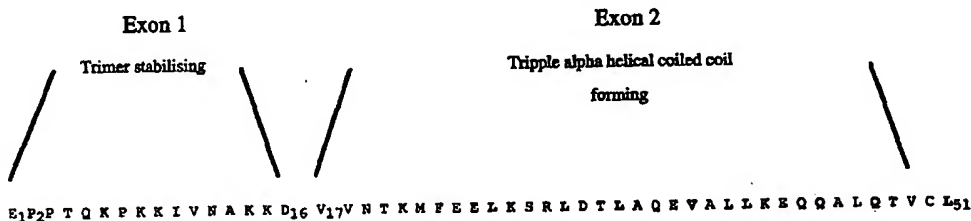


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(54) Title: TRIMERISING MODULE



(57) Abstract

The present invention relates to the design of trimeric polypeptides using polypeptide structural elements derived from the tetranectin protein family, and their use in rational *de novo* design and production of multi-functional molecules including the application of the multi-functional molecules in protein library technology, such as phage display technology, diagnostic and therapeutic systems, such as human gene therapy and imaging. The trimeric polypeptides being constructed as a monomer polypeptide construct comprising at least one tetranectin trimerising structural element (TTSE) which is covalently linked to at least one heterologous moiety, said TTSE being capable of forming a stable complex with two other TTSEs; or as an oligomer which is comprised of two monomer polypeptide constructs as mentioned above, and which comprises three TTSEs or a multiplicity of three TTSEs, or which is comprised of three monomer polypeptide constructs.

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TRIMERISING MODULE

The present invention relates to the design of trimeric polypeptides using polypeptide structural elements derived from the tetranectin protein family, and their use in rational *de novo* design and production of multi-functional molecules including the application of the multi-functional molecules in protein library technology, such as phage display technology, diagnostic and therapeutic systems, such as human gene therapy and imaging.

10 BACKGROUND OF THE INVENTION

Tetranectin is a Ca^{2+} -binding trimeric C-type lectin which is present in blood plasma and from the extracellular matrix of certain tissues. The tetranectin group of proteins comprises tetranectin isolated from man and from mouse and the highly related C-type lectin homologues isolated from the cartilage of cattle (Neame and Boynton, database accession number PATCHX:u22298) and from reef shark (Neame et al., 1992, Neame et al., 1996 and database accession number p26258 and PIR2:A37289).

20 The mature tetranectin polypeptide chain of 181 amino acid residues is encoded in three exons as shown by molecular cloning and characterisation of the gene (Berglund & Petersen, 1992; Wewer & Albrechtsen, 1992). Exon 3 of the human tetranectin gene encodes a separate functional and structural unit, a single long-form so-called carbohydrate recognition domain (CRD), with three intra-chain disulphide bridges. The tetranectin CRD is considered to belong to a distinct class of C-type lectins (Day, 1994) clearly related to C-type lectins by sequence homology, conservation of disulphide topology (Fuhlendorff et al, 1987) and by the presence of an almost conserved suit of amino acid residues known to be involved in binding of calcium ions.

A published poster (Holtet et al 1996) has proposed tetranectin to be a trimer and that trimerisation is governed by the peptide encoded by exon 1. The peptide encoded by exon 1 was proposed to be "necessary and sufficient to govern trimerisation" whereas the polypeptide encoded by exon 2 was proposed as being "involved in lysine-sensitive binding to plasminogen".

Tetranectin was first identified as a plasma protein binding to plasminogen by binding to the kringle-4 domain of plasminogen. Recent unpublished results (Graversen et al., manuscript for PNAS) proves (1) that the site in tetranectin involved in binding to plasminogen resides entirely in the CRD-domain (encoded by exon 3), (2) that binding is calcium sensitive, and (3) that the kringle-4 binding site in tetranectin overlaps the putative carbohydrate binding site of the CRD domain. Hence, there is now surprising definitive evidence that TN exons 1 and 2, i.e. the trimerisation unit in TN does not exhibit any plasminogen-binding affinity. Accordingly, an artificial protein containing a TTSE unit as part of its architecture is not expected to interact with plasminogen or plasmin due to properties inherited from tetranectin.

Tetranectin has also been reported to bind to sulfated polysaccharides like heparin (Clemmensen (1989) Scand J. Clin. Lab. Invest. vol 49:719-725). We have new results showing that the CRD domains of tetranectin are not involved in this protein-polysaccharide interaction. In fact, the site in tetranectin is located in the N-terminal region of exon 1 and may be abolished by removal or mutagenesis of N-terminal lysine residues (Graversen et al., manuscript), processes that do not inhibit trimerisation. TTSEs that include most or all of TN exon 1 therefore confer an affinity for sulfated polysaccharides to any designed protein which encompasses such a TTSE as part of its structure. If desired, however, this affinity can be reduced or abolished by N-terminal truncation or mutagenesis of lysine residues in the part of

the TTSE that corresponds to the N-terminal 8-10 amino acid residues of exon 1 (Graversen et al., unpublished).

With respect to gene therapy which is also within the scope of the present invention, there is only a limited number of basic strategies for gene therapy which show some promise in preclinical models so far. The two major strategies e.g. for the treatment of malignant tumors are cytokine-gene aided tumor vaccination and selective prodrug activation. Whereas the first strategy relies on the strong immunostimulatory effect of a relatively small number of genetically modified cytotoxic T cells or tumor cells, the second one is based on conversion of a nontoxic prodrug into a toxic product by an enzyme-encoding gene where the toxic effect is exerted also on non-transduced dividing tumor cells due to a so-called bystander effect. Alternatively, strategies can be envisaged where the malignant phenotype of a cell is reversed by either inactivating an oncogene or reestablishing an inactivated tumor suppressor gene. In both cases, highly efficient gene transfer to the cells in a tumor is required. Although high efficiencies of gene transfer can be obtained *in vitro* and even *in vivo* under certain circumstances, correction of the malignant phenotype by reversing the major oncogenic change in the tumor cells is unlikely to result in normal cells. Thus, selective induction of tumor cell death by use of the present invention would be preferable, and the development of methods enabling such induction will be of great importance.

A major problem in connection with the gene therapy is the incorporation of foreign material into the genome. Viruses, however, have only been partially successful in overcoming this problem. Hence the initial efforts at gene therapy are still directed towards engineering viruses so that they could be used as vectors to carry therapeutic genes into patients. In the still very immature *in vivo* method of somatic gene therapy, where a vector could be injected directly into the bloodstream, or more preferably by transmucosal delivery, the present invention may be utilized due to the surprising number of ways the gene therapy may be targeted.

For many gene-therapy applications in the future, it is probable that a synthetic hybrid system will be used that incorporates engineered viral component for target-specific binding and core entry, immunosuppressive genes from various viruses and some mechanism that allows site-specific integration, perhaps utilizing AAV sequences or an engineered retroviral integrase protein. In addition, regulatory sequences from the target cell itself will be utilized to allow physiological control of expression of the inserted genes. All these components would be assembled in vitro in a liposome-like formulation with additional measures taken to reduce immunogenicity such as concealment by PEG

As mentioned, one of the current problems in gene therapy is the efficient delivery of nucleic acids to as many as possible of a specific population of cells in the body, and it is often not possible to find e.g. an appropriate viral vector that will find that particular cell population efficiently and selectively (Review on aspects of gene therapy: Schaper, W & Ito, W.D. Current Opinion in Biotechnology, 1996, vol. 7, 635-640. Nature Biotechnology 1998 vol 16 is an entire volume dedicated to protein- and gene delivery).

Given the possibility of in vitro generation of a human antibody against virtually any target antigen by phage technology, it follows that TTSEs, where one of the subunits is modified with a membrane integrating or associating entity, may be used as a practicable tool for generating a viral, bacterial or preferentially artificially assembled liposomal vehicle that will allow selective delivery of the contained material by infection or transfection of any cell population to which such a specific antibody may be generated. Moreover vehicles may, with the use of TTSEs, be individualised by selection of patient specific antibodies or by assembling TTSE units conjugated with scFvs selected from an ensemble of antibodies selected by the particular markers of the disease.

SUMMARY OF THE INVENTION

It has surprisingly been found by the present inventors that the human tetranectin polypeptide (and derivatives thereof) is capable of forming very stable trimers which have a number of advantageous characteristics and uses. Notably, the tetranectin molecule includes a trimerising structural element which can be used as carrier of other chemical entities, thereby providing a carrier molecule of a hitherto unseen versatility.

10 Prior published knowledge in the field of providing trimerising polypeptides of choice includes the disclosure in WO 95/31540 by Hoppe and Reid which describes a trimerisation module derived from collectin coiled coil structures and its application in engineering of artificially trimerised proteins. Several interesting areas of application are common to that patent publication and to the present disclosure. However, in several ways the properties of the trimerisation modules derived from the tetranectin protein family as disclosed herein are markedly different in fundamental architecture and represent surprisingly improved properties in comparison with the collectin trimerisation unit:

(1) Although the spatial structures of both trimerisation modules at a superficial level appear as similar in that both are ternary coiled coil structures of roughly equivalent spatial size the structural basis for adopting this spatial configuration is markedly distinct between the two groups of proteins. In fact, it is so distinct that the common belief prior to the work of Holtet et al. on cross-linking of human tetranectin (Holtet et al., 1996) was that this family of proteins were tetrameric (hence the name). Accordingly, the sequences of the tetranectin family of trimerisation modules does not conform to the declared common motif delineated for the collectin family (WO 95/31540, page 8).

(2) The thermal stability of the tetranectin trimerisation module (as shown in the examples) is such that the trimer can be shown to exist even at about 60°C (Example 4, trimerised tetranectin) or at about 70°C (Example 3, trimerised ubiquitin), whereas a collectin trimer unit falls apart at about 50-55°C (WO 95/31540, Example 1, page 36 therein).

(3) Whereas it remains uncertain whether the collectin trimerisation domain possibly allows attachment of fusion partners at C-terminal ends of the trimerisation module, and whereas no example has been reported of successful or claimed successful attachment of a foreign protein (except for the GST fusion partner) to the N-terminal region of the collectin trimerisation module, the information disclosed herein demonstrates that the tetranectin trimerisation module is more versatile in that it allows attachment of foreign proteins to either, as well as to both, terminus or termini simultaneously (Examples 1-4). This has important consequences as the tetranectin trimerisation module may be deployed to construct molecules that are able to interact (each end with a binding valency up to 3) simultaneously with two bulky interaction partners like e.g. cell surfaces.

(4) The virtual absence of subunit exchange between monomers of a trimer that has been trimerised using the tetranectin trimerisation modules disclosed herein is by first principles of thermodynamics correlated with the surprisingly high thermal stability of the complex. It will hence be apparent that the advantages inherent to the "pick-and-mix" applications of the technology, as disclosed herein, may be used to much greater advantage because of the much longer shelf life expected for the heterofunctional products of the present invention.

The polypeptide constructs CIIH6FXTN123, H6FXTN123, H6FXTN12, and H6FXTN23 which all involve parts of the tetranectin molecule have previously been prepared (cf. e.g. WO 94/18227) but these constructs have all been provided with a view to

facilitating expression and/or purification of the tetranectin derived moiety of the constructs. To the best of the inventors' knowledge no publications exist which reports any use of tetranectin derivatives as "building blocks" to which
5 other chemical moieties advantageously could be coupled.

Hence, in its broadest aspect the present invention relates to a monomer polypeptide construct comprising at least one tetranectin trimerising structural element (hereinafter designated a TTSE) which is covalently linked to at least one
10 heterologous moiety, said TTSE being capable of forming a stable complex with two other TTSEs, with the proviso that the heterologous moiety is different from any of the fusion proteins CIIH6FXTN123, H6FXTN123, H6FXTN12, H6FXTN23, the sequences of which are shown in SEQ ID NOs: 24-27. It is
15 preferred that the heterologous moiety is one which does not exclusively facilitate expression and/or purification of the monomer polypeptide construct.

The invention further relates to oligomeric molecules comprised of at least two of such monomer polypeptide constructs, and the invention also relates to methods of preparing the monomer polypeptide constructs and the oligomers. The invention further relates to a kit comprising monomer polypeptide constructs in separate packages, ready for use in
25 a "pick-and-mix" approach for use of the monomers. This pick and mix approach is for use in therapeutic as well as for diagnostic use. Eg. having a tumor with a known and specific epitope to which an antibody is available. The kit may then comprise a first TTSE conjugated to an relevant antibody, a
30 second component which may comprise a TTSE coupled to an imaging compound. In a second aspect the second component may comprise a drug or a prodrug relevant for treating the tumor. In a still further aspect a third TTSE component being a monitoring compound, e.g. indicative of the progress of the
35 targeting.

As is clear from this illustration the present invention relates to a large amount of combinations allowing an individual design for a large number of circumstances.

Finally, the invention also pertains to fragments which include nucleic acid sequences which encode the monomer polypeptide constructs, as well as to vectors and cells containing these nucleic acid fragments.

LEGENDS TO THE FIGURES

Fig. 1: Amino acid sequence of the amino terminal region of tetranectin.

Amino acid sequence (in one letter code) from E1 to L51 of tetranectin (SEQ ID NO: 7). Exon 1 comprises residues E1 to D16 and exon 2 residues V17 to V49, respectively. The alpha helix extends beyond L51 to K52 which is the C-terminal amino acid residue in the alpha helix.

Fig. 2: Alignment of the amino acid sequences of the trimerising structural element of the tetranectin protein family. Amino acid sequences (one letter code) corresponding to residue V17 to K52 comprising exon 2 and the first three residues of exon 3 of human tetranectin (SEQ ID NO: 7); murine tetranectin (Sørensen et al., Gene, 152: 243 -245, 1995); tetranectin homologous protein isolated from reef-shark cartilage (Neame and Boynton, 1992,1996); and tetranectin homologous protein isolated from bovine cartilage (Neame and Boynton, database accession number PATCHX:u22298). Residues at a and d positions in the heptad repeats are listed in boldface. The listed consensus sequence of the tetranectin protein family trimerising structural element comprise the residues present at a and d positions in the heptad repeats shown in the figure in addition to the other conserved residues of the region. "hy" denotes an aliphatic hydrophobic residue.

Fig. 3: Construction of the expression plasmids pTH6FXtripa and pTH6FXtripb.

The amplified DNA fragments tripa and tripb harbouring the tetranectin amino acid sequence (SEQ ID NO: 7) from E1 to T48 and E1 to K52, respectively, fused in the 5' end to nucleotide sequences encoding a FX_a cleavage site IQGR (SEQ ID NO: 4) and the recognition sites for the restriction endonucleases BglII and KpnI, were cut with the restriction enzymes BclI and HindIII and ligated into the BamHI and HindIII sites of the expression plasmid pT7H6 (Christensen et al., 1991) using standard procedures.

Fig. 4: Predicted amino acid sequence of the fusion proteins H6FXtripa (SEQ ID NO: 28) and H6FXtripb (SEQ ID NO: 29) encoded by the expression plasmids pTH6FXtripa and pTH6FXtripb, respectively.

Fig. 5: Construction of the expression plasmids pTH6FXTN123 and pTCIIH6FXTN123.

The amplified DNA fragment corresponding to the full length, mature tetranectin monomer (SEQ ID NO: 7) from E1 to V181 fused in the 5' end to nucleotide sequences encoding a FX_a cleavage site IEGR (SEQ ID NO: 10) was cut with the restriction enzymes BamHI and HindIII and ligated into the corresponding sites of the expression plasmids pT7H6 (Christensen et al., 1991) and pTCIIH6 using standard procedures. pTCIIH6 was derived from pT7H6 by substitution of the NdeI - HindIII fragment of pT7H6 with the NdeI - HindIII fragment of pLcII (Nagai and Thøgersen, 1987) encoding the first 32 residues of the lambda cII protein MVRANKRNEALRIESALLNKIAMLGTEKTAEG (SEQ ID NO: 11) fused in the 3' end to a nucleotide sequence encoding the H6 sequence GSHHHHHHGS (SEQ ID NO: 12).

Fig. 6: Predicted amino acid sequence of the fusion proteins H6FXTN123 (SEQ ID NO: 25) and CIIH6FXTN123 (SEQ ID NO: 24) encoded by the expression plasmids pTH6FXTN123 and pTCIIH6FXTN123, respectively.

Fig. 7: Construction of the expression plasmids pTH6FXTN12, pTH6FXTN23, and pTH6FXTN3.

The amplified DNA fragments corresponding to the tetranectin derivatives TN12 and TN3 from E1 to V49 and A45 to V181, respectively (SEQ ID NO: 7) fused in the 5' end to nucleotide sequences encoding the FX_a cleavage site IEGR (SEQ ID NO: 10) was cut with the restriction enzymes BamHI and HindIII and ligated into the corresponding sites of the expression plasmids pT7H6 (Christensen et al., 1991) using standard procedures. The amplified DNA fragment corresponding to the tetranectin derivative TN23 from V17 to V181 (SEQ ID NO: 7) fused in the 5' end to nucleotide sequences encoding the FX_a cleavage site IQGR (SEQ ID NO: 4) was cut with the restriction enzymes BamHI and HindIII and ligated into the corresponding sites of the expression plasmids pT7H6 (Christensen et al., 1991) using standard procedures.

Fig. 8: Predicted amino acid sequence of the fusion proteins H6FXTN12 (SEQ ID NO: 26), H6FXTN23 (SEQ ID NO: 27), and H6FXTN3 (SEQ ID NO: 30) encoded by the expression plasmids pTH6FXTN12, pTH6FXTN23, respectively.

Fig. 9: Gel filtration analysis of TN123, TN23, and TN3. Analytical gel filtration of the recombinant tetranectin derivatives TN123, TN23, and TN3 were performed on a Superose 12 HR 10/30 column (Pharmacia, Sweden) with a total volume of 25 ml in 100 mM NaCl and 50 mM Tris-HCl pH 8 and a flow rate of 0.2 ml/min. Vertical bars at peak maxima identify elution profiles for each of the three proteins.

Fig. 10: Cross-linking analysis of TN123 and CIIH6FXTN123. Samples of TN123, CIIH6FXTN123 and mixtures of both were incubated with DMSI and analyzed by SDS-PAGE (12% gel). Before addition of DMSI, protein mixtures were subjected to subunit exchange by incubation at 70°C for varying length of time. Protein marker of 94, 68, 43 and 30 kDa, top to bottom (lane M). CIIH6FXTN123 fusion protein (lane 1). TN123 (lane 2). DMSI treated CIIH6FXTN123 (lanes 3 and 6). DMSI-treated

TN123 (lane 4). Identical samples of DMSI treated mixtures of CIIH6FXTN123 and TN123 without heat exposure (lanes 5 and 7) and heat treated for 2.5 sec, 15 sec, 2.5 min. and 10 min., respectively, before treatment with DMSI (lanes 8-11).

- 5 Fig. 11: Cross-linking analysis of the recombinant tetranectin derivatives TN123, TN23, TN3, and H6FXTN12. The recombinant proteins TN123, TN23, TN3, H6FXTN12 or mix-
tures of TN123 and each of the other were analyzed by SDS-
PAGE. Protein marker of 94, 68, 43, 30, 20, and 14.4 kDa, top
10 to bottom (lane M). TN123 cross-linked with DMSI (lane 1). TN123 and H6-rTN12 cross-linked with DMSI without and with heat treatment at 70°C for two min. (lanes 2 and 3). H6FXTN12 cross-linked with DMSI (lanes 4 and 5). Mixture of TN123 and H6FXTN12, no cross-linking (lane 6). Cross-linking of TN123
15 and TN23 without and with heat treatment at 70°C for two min. (lanes 7 and 8). Cross-linking of TN23 (lane 9). Mixture of TN123 and TN23 without cross-linking (lane 10). TN123 cross-linked by DMSI (lane 11). Cross-linking of TN123 and TN3 without and with heat treatment for two min. (lanes 12 and
20 13). Cross-linking of TN3 (Lane 14). Mixture of TN123 and TN3, no cross-linking (lane 15).

Fig. 12: Cross-linking based analysis of the trimer thermal stability.

- In parallel experiments TN123 and the fusion protein
25 H6FXtripb-UB (SEQ ID NO: 31) were cross-linked with DMSI at different temperatures and the samples analyzed by SDS-PAGE. Protein marker of 94, 68, 43, 30, 20, and 14.4 kDa, top to bottom (lane M). TN123 without cross-linking (lane 1). TN123 cross-linked with DMSI for 15 min. at 37°C, 50°C, 60°C, and
30 70°C (lanes 2 to 5), respectively. The fusion protein H6FXtripb-UB (SEQ ID NO: 31) without cross-linking (lane 6). H6FXtripb-UB cross-linked with DMSI for 15 min. at 37°C, 50°C, 60°C, and 70°C (lanes 7 to 10), respectively and H6FXtripb-UB incubated at 70°C for 15 min. (lane 11).

Fig. 13: Construction of the expression plasmid pTH6FXtripb-UB.

The amplified DNA fragment comprising the nucleotide sequence (SEQ ID NO:16) encoding the ubiquitin amino acid sequence
5 (SEQ ID NO: 19) from Q2 to G76 was cut with the restriction enzymes BclI and HindIII and ligated into the BamHI and HindIII sites of the expression plasmid pT7H6FXtripb (Example 1) using standard procedures.

Fig. 14: Predicted amino acid sequence of the fusion protein
10 H6FXtripb-UB (SEQ ID NO: 31) encoded by the expression plasmid pTH6FXtripb-UB.

Fig. 15: Construction of the expression plasmid pTH6FXscFV (CEA6)tripb.

The DNA fragment, amplified with the primer pair SEQ ID NOs:
15 21 and 22, comprising the nucleotide sequence SEQ ID NO: 20 encoding the single chain antibody CEA6, scFV (CEA6), amino acid sequence from Q1 to A261 was cut with the restriction enzymes BamHI and KpnI and ligated into the BglII and KpnI sites of the expression plasmid pT7H6FXtripb (Example 1)
20 using standard procedures.

Fig. 16: Predicted amino acid sequence of the fusion protein H6FXscFV(CEA6)tripb encoded by the expression plasmid pTH6FXscFV(CEA6)tripb.

Fig. 17: Construction of the expression plasmid
25 pTH6FXtripbscFX(CEA6).

The DNA fragment, amplified with the primer pairs having SEQ ID NO: 21 and 23, comprising the nucleotide sequence (SEQ ID NO: 20) encoding the single chain antibody CEA6, scFV (CEA6), amino acid sequence from Q1 to A261 was cut with the restriction
30 enzymes BamHI and HindIII and ligated into the BamHI and HindIII sites of the expression plasmid pT7H6FXtripb (Example 1) using standard procedures.

Fig. 18: Predicted amino acid sequence of the fusion protein H6FXtripbscFv(CEA6) encoded by the expression plasmid pH6FXtripbscFv(CEA6).

Fig. 19: Construction of the expression plasmid pTH6FXscFv(CEA6)tripbscFX(CEA6).
The DNA fragment, amplified with the primer pair SEQ ID NO: 21 and 23, comprising the nucleotide sequence (SEQ ID NO: 20) encoding the single chain antibody CEA6, scFV (CEA6), amino acid sequence from Q1 to A261 was cut with the restriction enzymes BamHI and HindIII and ligated into the BamHI and HindIII sites of the expression plasmid pT7H6FXscFv(CEA6)tripb (Example 4) using standard procedures.

Fig. 20: Predicted amino acid sequence of the fusion protein H6FXscFv(CEA6)tripbscFv(CEA6) (SEQ ID NO: 34) encoded by the expression plasmid pH6FXscFv(CEA6)tripbscFv(CEA6).

Fig. 21: Cross-linking analysis of the H6FXtripbscFv(CEA6) fusion protein (SEQ ID NO: 33).
In parallel experiments the fusion proteins H6FXtripbscFv(CEA6) (SEQ ID NO: 33) and TN123 were cross-linked at room temperature for 30 min. with 0 mg/ml, 0.5 mg/ml, 1.0 mg/ml, 1.5 mg/ml, and 2.0 mg/ml of DMSI, respectively. Lane 1: H6FXtripbscFv(CEA6) without DMSI, H6FXtripbscFv(CEA6) with 0.5 mg/ml DMSI (lane 2), H6FXtripbscFv(CEA6) with 1.0 mg/ml DMSI (lane 3), H6FXtripbscFv(CEA6) with 1.5 mg/ml DMSI (lane 4) and H6FXtripbscFv(CEA6) with 2.0 mg/ml DMSI (lane 5). Protein marker of 94, 68, 43, 30, 20, and 14.4 kDa, top to bottom (lane M). Lane 6: TN123 without DMSI, TN123 with 0.5 mg/ml DMSI (lane 7), TN123 with 1.0 mg/ml DMSI (lane 8) TN123 with 1.5 mg/ml DMSI (lane 9) and TN123 with 2.0 mg/ml DMSI (lane 10).

DETAILED DISCLOSURE OF THE INVENTION

The term "trimerising structural element" (TTSE) used in the present description and claims is intended to refer to the portion of a polypeptide molecule of the tetranectin family which is responsible for trimerisation between monomers of the tetranectin polypeptide. The term is also intended to embrace variants of a TTSE of a naturally occurring tetranectin family member, variants which have been modified in the amino acid sequence without adversely affecting, to any substantial degree, the trimerisation properties relative to those of the native tetranectin family member molecule. Specific examples of such variants will be described in detail herein, but it is generally preferred that the TTSE is derived from human tetranectin, murine tetranectin, C-type lectin of bovine cartilage, or C-type lectin of shark cartilage. Especially preferred is monomer polypeptide constructs including at least one TTSE derived from human tetranectin.

The 49 residue polypeptide sequence encoded by exons 1 and 2 of tetranectin (Fig. 1) appears to be unique to the tetranectin group of proteins (Fig. 2) as no significant sequence homology to other known polypeptide sequences has been established. In preparation for experimental investigations of the architecture of tetranectin a collection of recombinant proteins was produced, the collection including complete tetranectin, the CRD domain (approximately corresponding to the polypeptide encoded by exon 3), a product corresponding to the polypeptide encoded by exons 2+3, a product corresponding to exons 1+2 (Holtet et al., 1996; Example 2). As detailed in Example 2 we now know differently: tetranectin is indeed a trimer, but the exon 2 encoded polypeptide is in fact capable of effecting trimerisation by itself as evidenced by the observation that the recombinant protein corresponding to exons 2+3 is in fact trimeric in solution.

3D-structure analysis of crystals of full-length recombinant tetranectin (Nielsen et al., 1996; Nielsen, 1996; Larsen et al., 1996; Kastrup, 1996) has shown that the polypeptide

encoded in exon 2 plus three residues encoded in exon 3 form a triple alpha helical coiled coil structure.

From the combination of sequence and structure data it becomes clear that trimerisation in tetranectin is in fact generated by a structural element (Fig. 2), comprising the amino acid residues encoded by exon two and the first three residues of exon 3 by an unusual heptad repeat sequence, that apparently is unique to tetranectin and other members of its group: This amino acid sequence (Fig. 2) is characterised by two copies of heptad repeats (abcdefg) with hydrophobic residues at a and d positions as are other alpha helical coiled coils. These two heptad repeats are in sequence followed by an unusual third copy of the heptad repeat, where glutamine 44 and glutamine 47 not only substitute the hydrophobic residues at both the a and d position, but are directly involved in the formation of the triple alpha helical coiled coil structure. These heptad repeats are additionally flanked by two half-repeats with hydrophobic residues at the d and a position, respectively.

The presence of beta-branched hydrophobic residues at a or d positions in alpha helical coiled coil are known to influence the state of oligomerisation. In the tetranectin structural element only one conserved valine (number 37) is present. At sequence position 29 in tetranectin no particular aliphatic residue appears to be preferred.

In summary, it is apparent that the triple stranded coiled coil structure in tetranectin to a large extent is governed by interactions that are unexpected in relation to those characteristic among the group of known coiled coil proteins.

The TTSEs form surprisingly stable trimeric molecules. (Examples 2, 3 and 4). The experimental observations, that (1) a substantial part of the recombinant proteins exists in the oligomeric state of - and can be cross-linked as - trimeric molecules even at 70°C and (2) that exchange of

monomers between different trimers can only be detected after exposure to elevated temperature are evidence of a extremely high stability of the tetranectin trimerising structural element. This feature must be reflected in the amino acid sequence of the structural element. In particular, the presence and position of the glutamine containing repeat in the sequential array of heptad repeats is, together with the presence and relative position of the other conserved residues in the consensus sequence (Fig. 2), considered important for the formation of these stable trimeric molecules. For most practical uses the cysteine residue 50 should be mutagenized to serine, threonine, methionine or to any other amino acid residue in order to avoid formation of an unwanted inter-chain disulphide bridge, which eventually would lead to uncontrolled multimerisation, aggregation and precipitation of a polypeptide product harbouring this sequence.

In particular in conjunction with the trimer-stabilising exon 1 encoded polypeptide (tetranectin residues 1 to 16, see Example 2), the tetranectin trimerising structural element is a truly autonomous polypeptide module retaining its structural integrity and propensity to generate a highly stable homotrimeric complex whether it is attached or not by a peptide bond at either or at both termini to other proteins. This unique property is demonstrated in the accompanying examples, which provide experimental proof, that polypeptide sequences derived from heterologous proteins may readily be trimerised when joined as fusion proteins to the tetranectin trimerising structural element. This remains valid irrespective of whether the heterologous polypeptide sequences are placed amino-terminally or carboxy-terminally to the trimerising element allowing for the formation of one molecular assembly containing up to six copies of one particular polypeptide sequence or functional entities, or the formation of one molecular assembly containing up to six different polypeptide sequences, each contributing their individual functional property.

Since three TTSEs of naturally occurring human tetranectin forms up a triple alpha helical coiled coil, it is preferred that the stable complex formed by the TTSEs of the invention also forms a triple alpha helical coiled coil.

- 5 The "tetranectin family" are polypeptides which share the consensus sequence shown in Fig. 2 or a sequence which are homologous at sequence level with this consensus sequence. Hence, monomer polypeptide constructs of the invention are preferred which comprise a polypeptide sequence which has at
10 least 68% sequence identity with the consensus sequence shown in Fig. 2, but higher sequence identities are preferred, such as at least 75%, at least 81%, at least 87%, and at least 92%.

By the term "heterologous moiety" is herein meant any chemical entity which can be linked covalently to a TTSE and to
15 which the TTSE is not natively covalently bound. Hence, the heterologous moiety can be any covalent partner moiety known in the art for providing desired binding, detection, or effector properties. The heterologous moiety can be a ligand
20 binding structure such as a receptor molecule or the ligand binding part of a receptor molecule, an antibody, an antigen binding antibody fragment, or a molecule having antibody characteristics such as e.g. the "diabodies" described in EP-A-0 672 142, or other ligand binding molecules such as
25 avidin or streptavidin, or a lectin; a toxin such as ricin; a detectable label such as a fluorescence labelled molecule, a radioactively labelled molecule, an enzymatically labelled molecule; an *in situ* activatable substance, such as a molecule which can be induced by a magnetic field or by
30 radiation to be radioactively or chemically active; an enzyme such as a peroxidase; a radioactive moiety such as a γ -, α -, β^- -, or β^+ -emitting molecule, e.g. a molecule comprising one or more radioactive isotopes selected from ^{14}C , ^3H , ^{32}P , ^{33}P , ^{25}S , ^{38}S , ^{36}Cl , ^{22}Na , ^{24}Na , ^{40}K , ^{42}K , ^{43}K , and any isotopes
35 conventionally utilized for the purposes of facilitating detection of probes or the purposes of providing localized

radiation so as to effect cell death; a cytokine such as an interferon or a leukotriene; PNA; a non-proteinaceous polymer such as a polymeric alkaloid, 11.a polyalcohol, a polysaccharide, a lipid and a polyamine; a photo cross-
5 linking moiety, i.e. a chemical entity which effects cross-linking upon photo-activation; and a group facilitating conjugation of the monomer polypeptide construct to a target.

The heterologous moiety is preferably covalently linked to the TTSE by via a peptide bond to the N- or C-terminus of the
10 TTSE peptide chain, via a peptide bond to a side chain in the TTSE or via a bond to a cysteine residue, but any way of coupling covalently heterologous material to a polypeptide chain will be useful. The skilled person will know of such possibilities, e.g. by consulting the teachings of WO
15 95/31540 in this regard which are hereby incorporated by reference.

However, one interesting aspect of the invention relates to a monomer polypeptide construct of the invention comprising two heterologous moieties which are linked via peptide bonds to
20 the N- and C-terminus, respectively. This approach introduces a number of possibilities in terms of e.g. linking larger entities with oligomers of the invention by having specific activities coupled to each end of the monomers (as explained in detail below, the oligomers of the invention may also
25 utilise a version of this principle, where e.g. one N-terminus and one C-terminus of an oligomer are linked via peptide bonds to independent heterologous moieties).

In general, a complex between two or three monomers are described in the following way: three monomers having one
30 TTSE each forms a trimer designated (1+1+1), whereas a dimer formed between a monomer with two TTSEs and a monomer with one TTSE is designated (1+2). Other (undesired) trimers can of course be formed, e.g. (2+2+1), where two TTSEs are not "in use", but it is preferred that the oligomers of the
35 invention use all of their available TTSEs during complex

formation. It should also be noted that the term "monomer polypeptide construct" is meant to designate a single polypeptide chain which may or may not have non-peptide groups coupled covalently to the polypeptide chain, whereas
5 "dimeric polypeptide" or "dimer", "trimeric polypeptide" or "trimer" and "oligomer" (i.e. a dimer or trimer) in the present context are meant to designate non-covalent complexes of monomer polypeptide constructs. I.e., the traditional definitions of monomers and multimers do not apply in the
10 present specification and claims.

The TTSE as exemplified by exon 2 or exons 1 and 2 of human tetranectin, preferably so modified to allow only heterotrimerisation between dissimilar (1+1+1) or (1+2) (cf. the below discussion) subunits may be deployed as a general
15 affinity mediator, which can be coupled chemically to each member of a selection of target molecules. After such conjugation with TTSE the target molecules may be homo- or heterotrimerised as desired for any application. Similar deployment of dimerisation, using as one partner a polypeptide harbour-
20 ing two TTSE sequences in-line, separated by a linker sequence of suitable length and character, may be yet more advantageous, as in such case absolute control of stoichiometry in complex formation would be possible. Thus, an important embodiment of the invention is a monomer
25 polypeptide construct of the invention comprising 2 TTSEs which are covalently linked by a spacer moiety which allows both of the 2 TTSEs to take part in complex formation with a third TTSE not being part of the monomer polypeptide construct, but equally important is the embodiment of the invention where the monomer polypeptide construct comprises one
30 single TTSE, so as to allow trimerisation between three monomers and hence providing the optimum degree of versatility with respect to the number of functional units which can be easily incorporated into one single complex.

35 In the embodiments where two TTSEs are present in the same monomer it is preferred that the spacer moiety has a length

and a conformation which favours complex formation involving both of the two TTSEs which are covalently linked by the spacer moiety. In this way, problems arising from undesired formation of trimers of the formats (2+1+1), (2+2+2), and
5 (2+2+1) (wherein only one TTSE of each monomer participates in complex formation) can be diminished. Design and preparation of suitable spacer moieties are known in the art and are conveniently effected by preparing fusion polypeptides having the format TTSE¹-Spacer-TTSE², where the spacer moiety is a
10 polypeptide fragment (often a relatively inert one), so as to avoid undesired reactions between the spacer and the surroundings or the TTSEs.

One typical scenario, where such modification may be advantageous is the case of immunological detection where a chemical
15 conjugate of an antibody with enzymes such as peroxidase is used for in situ staining purposes in tissue or on western blots.

A similar, but yet different, application example would be the deployment of TTSE to mediate conjugation of e.g. alkaline
20 phosphatase and an oligonucleotide which would allow in situ identification of a given mRNA in a tissue sample concurrently with identification of any other mRNA molecule e.g. by interconnection of a second appropriate oligonucleotide and a signalling/visualisation molecule using e.g. the
25 biotin - avidin/streptavidin affinity pair for conjugation. The point of having two or more selective affinity systems available for conjugating oligonucleotide probes and detector molecules is that as many different sequences may be detected simultaneously as there are affinity sets available.

30 In terms of chemistry required to exploit TTSE as a conjugating affinity-contributing agent, the peptide corresponding to exon 2 will have a sufficient affinity for most purposes, but incorporation of all, or some segment of the exon 1 polypeptide will serve to increase affinity and stability.
35 The properties of tetranectin mutants in which many of hydro-

philic (e.g. lys and glu) residues that are largely exterior in the coiled coil structure have been replaced with alanine appear similar to the native protein, suggesting that is indeed possible without interfering very much with stability of the trimeric structure to replace all glu, asp and lys residues by a combination of gln, asn, arg or ala, and thereby generate a sequence that, as an N-terminally blocked synthetic peptide, would be very easy to convert into a chemically stable active-ester component, e.g. an N-hydroxy succinimide ester of an acetylated peptide, that could react with (and thereby couple to) any exposed lysine side chain in a target molecule of interest. Such peptide synthesis, activation and coupling chemistry will be readily designed and applied by a person skilled in the art of peptide chemistry, as will indeed any other conjugation chemistry, like the attachment and use of photo-activatable moieties like e.g. phenyl azides.

In conclusion, it seems that the most important structure in native TTSE is the consensus sequence shown in Fig. 2, and that large variations in the polypeptide chain may be allowed. Hence, one advantageous embodiment of the monomer polypeptide construct of the invention is one where at least one amino acid residue selected from the group consisting of amino acid residue nos. 6, 21, 22, 24, 25, 27, 28, 31, 32, 35, 39, 41, 42, is/are substituted by any non-helix breaking amino acid residue, the amino acid residue numbering referring to amino acid residues in SEQ ID NO: 7. All these residues have been shown not to be directly involved in the intermolecular interactions which stabilises the trimeric complex between three TTSEs of native tetranectin monomers and it is therefore expected that these amino acids may be safely substituted with any amino acid which will not have an adverse effect on helix formation (notably proline, which introduces a rigid bend in a polypeptide chain).

Another advantageous embodiment of the monomer polypeptide construct of the invention is one which is free from any free

amino and/or carboxy groups. This would favour synthesis of a TTSE by means of solid or liquid phase peptide synthesis, since there would be no need of introducing any protective groups during peptide synthesis.

- 5 Since the consensus sequence of Fig. 2 is believed important and since this consensus sequence embraces the above-discussed heptad repeat, it is according to the invention preferred that the TTSE comprises a repeated heptad having the formula a-b-c-d-e-f-g (N to C), wherein residues a and d
10 generally are hydrophobic amino acids. However, since "a" and "d" in the third of the complete heptads of all known members of the tetranectin family are constituted of glutamine, it is most preferred that the TTSE comprises the heptad repeated 3
15 times and that the last occurrence of the heptad has a glutamine residue corresponding to residues a and d.

Since exon 2 of native members of the tetranectin family seems to contain the necessary elements to effect stable trimerisation, it is preferred that the monomer polypeptide construct is free of substantial parts of tetranectin which
20 is encoded by exon 3 and/or lacks substantial parts of tetranectin which is encoded by exon 1. However, since exon 1 encoded material seems to stabilise the trimeric native tetranectin, it is especially preferred that all or part of exon 1 is part of the monomer polypeptide construct, and it
25 also seems to be rational to include the first three amino acids encoded by exon 3, since these are known to take part of the formation of the native triple alpha helical coiled coil in human tetranectin.

One particularly interesting embodiment of the invention is
30 the possibility of designing oriented molecular assemblies, where one or more functional entities are located N-terminally to the trimerising element and one or more functional entities are located C-terminally to the element. Such types of design may be particularly advantageous where a certain
35 relative ratio is desired among the different functional

entities included in a specific molecular unit. Such type of design may in addition be used if one or more functional entities for either structural or functional reasons appear incompatible within the same construct. Such may be the case if one or more of the functional entities are expressed by large or bulky protein domains which for steric reasons might prevent formation of the trimeric molecular unit due to sterical constraints.

The possibility of constructing bi-polar three-way fusion proteins in which one functionality is placed N-terminally to the coiled coil structure and a different functionality is placed C-terminally is additionally advantageous in applications where large spatial separation between the two functionalities are desirable for optimal function. Examples of such application are e.g. the deployment of binding domains (e.g. antibody-derived binding modules) for recognition and binding to binding sites located at or close to large structures like cell membranes in cases where it is advantageous to allow for binding of the other end of the trimerised molecule to a different, but also bulky target.

Hence, as discussed above, the oligomers of the invention may be used to join e.g. bulky surfaces by the oligomer according to the invention comprising at least one heterologous moiety which is positioned N-terminally to a TTSE and at least one heterologous moiety which is positioned C-terminally to a TTSE. The two heterologous moieties can be either part of the same monomer polypeptide construct or parts of two separate monomer polypeptide constructs.

The extraordinarily high stability of any trimer containing the tetranectin trimerisation module under physiological buffer and temperature condition (i.e. absence of denaturant, temperature not exceeding 40°C) in combination with the ease by which exchange of monomer subunits between trimers can be effected by incubation at moderately elevated temperature or in the presence of denaturants provide for a unique opportu-

nity to deploy the trimerisation module as a vehicle to allow the construction of "pick-and-mix" conjugates prepared from previously fabricated collections of homotrimeric molecules. To illustrate the versatility of this design opportunity by way of theoretical example, let us assume that (1) a collection of twenty different antibody constructs (e.g. in the format of single-chain Fv) each of its own characteristic binding specificity, has been selected and then turned into homo-trimeric molecules by fusion to a tetranectin trimerisation module, and let us also assume that a set of twenty different effector molecules (e.g. toxin domains) have similarly been prepared and also conjugated to the tetranectin trimerisation module. A user provided with prefabricated collections of twenty different antibody constructs and twenty different toxin constructs - 40 different reagents in all - has the opportunity then to prepare 400 different toxin-antibody conjugates, simply by mixing a first preferred component from one reagent collection with a second preferred reagent from the other collection and then subject this binary mixture to conditions, i.e. gentle heating or incubation with a suitable level of denaturant, to accomplish subunit exchange among all trimeric molecular species in the mixture. After the subunit exchange step the desired heterobifunctional reagent will be present in the mixture as a major component of the mixture and may then be deployed as such to accomplish a given purpose or, alternatively apply a simple purification step to isolate his favoured heterofunctional binary reagent from any remaining mono-functional trimer species by a simple standard protein purification step, easily designed using standard techniques known in the field of protein purification.

A further enhancement of the versatility of the "pick-and-mix" technology may be achieved by including a specific affinity purification tag on each array of trimerisation module - probe/effector/indicator conjugate, fused directly in-line or, alternatively, fused via a cleavable linker (a polypeptide segment containing e.g. a factor X_a or an

enterokinase recognition/cleavage site) to the affinity tag. More specifically, if each of three libraries were tagged with affinity handles a, b and c, respectively, that were recognised by binding substances A, B and C, respectively, 5 pure heterotrimers, composed of one member of each library, could be obtained in a three-step affinity purification procedure designed to allow selective recovery of only such trimers that exhibit affinity for substances A and B and C. If, for any reason, subsequent removal of the affinity tags 10 were desirable, and the constructs had been prepared to include cleavable linkers, isolation of the pure heterotrimer, liberated from all affinity tags, could be accomplished by three further affinity purification steps, arranged to isolate only material that would bind to neither substance A 15 nor substance B nor substance C.

Obviously, the scope of "pick-and-mix" design of user-preparable heterofunctional complexes apply not only to the formation of binary hetero-functionality, but would apply by logic extension to the formation of ternary hetero-functionality: 20 To envisage the wealth of possibilities that are inherent to the concept of ternary hetero-functionality in a further theoretical example along the lines given above, three sets of reagent collections, each comprising 20 different functional characteristics, i.e. a collection of in toto 60 25 different homotrimers would allow "pick-and-mix" preparation of 8,000 different tri-functional molecules.

The basic tetranectin trimerisation module will, essentially indiscriminately, form homo- and hetero-trimers with any molecule that also contains a trimerisation module. For some 30 applications it may be advantageous to have available specially engineered derivatives of the tetranectin trimerisation module, which have been reengineered to disallow homotrimer formation and hence only allow hetero-trimerisation. Thus, an important embodiment of the monomer polypeptide 35 construct of the invention is constructed/reengineered so as to disfavour formation of complexes between identical TTSEs;

this also has the implication that oligomers of the invention can advantageously be comprised of monomer polypeptide constructs which are designed so as to disfavour formation of trimers including two monomer polypeptide constructs having
5 identical TTSEs. One way of disfavouring the formation of homotrimerisation would be by "knobs into holes" mutagenesis.

The design/reengineering may be accomplished by introduction of amino acid substitution at sites in the polypeptide intimately involved in the formation and stability of the trimer
10 and, simultaneously, in a different construct introduce a compensatory amino acid substitution, all in all removing symmetry between individual monomer components of the triple helical structure so that the structural complementarity profile only allows the formation of hetero-trimers, but is
15 incompatible with some or each of the homotrimer species.

A yet different way to deploy the tetranectin trimerisation module as a vehicle to accomplish rational formation of bifunctionalisation would require the interconnection of the C-terminus of one monomer to the N-terminus of a second
20 monomer in the triple-helical structure. The basic requirement for such an intervening polypeptide is, that allowed spatial distances between its N- and C-termini must be compatible with the spacing inherent to the structural requirements given by the architecture of the tetranectin trimerisation module. The construction of an intervening connecting
25 polypeptide allowed according to such criteria would be readily accomplished by an average person skilled in the art of protein engineering, as an ample collection of examples of the deployment of, usually flexible, spacer sequences are
30 known both in nature and in designed proteins. Due to the expected entropic contribution to interaction energy in a molecule in which two of the three tetranectin trimerisation module components are covalently tied together, such a molecule would show great preference for selecting any molecule
35 containing only a single copy of the tetranectin trimerisation

tion module component, as this selection would be energetically favoured. Hence, conjugation of one functional protein component to a suitably selected covalently dimerised tetra-nectin trimerisation module component and conjugation of a
5 different functional protein component to a single-copy element of the trimerisation sequence would provide for the preferential formation of a 1:1 bifunctional complex and suppression of formation of any other complex.

The monomers of the invention may be prepared by methods
10 generally known in the art, using exclusively or in combination the techniques of recombinant protein production, peptide synthesis (liquid phase or solid phase), and traditional chemical coupling of heterologous moieties to a peptide chain or to specific residues therein. Hence the
15 invention also relates to a method of preparing the monomer polypeptide construct of the invention, the method comprising

- isolating the monomer polypeptide construct from a culture comprising a host cell which carries and expresses a nucleic acid fragment which encodes the monomer
20 polypeptide construct,
- synthesizing, by means of chemical peptide synthesis, the monomer polypeptide construct and subsequently isolating the monomer polypeptide construct from the reaction mixture,
- 25 - preparing a TTSE in a culture comprising a host cell which carries and expresses a nucleic acid fragment which encodes the TTSE, subsequently linking covalently at least one heterologous moiety to the TTSE, and thereafter isolating the resulting monomer polypeptide construct, or
- 30 - synthesizing, by means of chemical peptide synthesis, a TTSE, subsequently linking covalently at least one heterologous moiety to the TTSE, and thereafter the

isolating the resulting monomer polypeptide construct from the reaction mixture,

and optionally subjecting the monomer polypeptide construct to further processing.

- 5 The nucleic acid fragment which is mentioned above is also a part of the invention and is defined as a nucleic acid fragment in isolated form which encodes a TTSE as defined herein or which encodes the polypeptide part of a monomer polypeptide construct according to the invention, with the
- 10 proviso that the nucleic acid fragment is different from one that encodes native members of the tetranectin family, and that the nucleic acid fragment is different from one that encodes any of the fusion proteins CIIH6FXTN123, H6FXTN123, H6FXTN12, H6FXTN23, the sequences of which are shown in SEQ
- 15 ID NOs: 24-27.

The above mentioned host cell (which is also a part of the invention) can be prepared by traditional genetic engineering techniques which comprises inserting a nucleic acid fragment (normally a DNA fragment) encoding the polypeptide part of a

20 monomer polypeptide construct of the invention into a suitable expression vector, transforming a suitable host cell with the vector, and culturing the host cell under conditions allowing expression of the polypeptide part of the monomer polypeptide construct. The nucleic acid fragment encoding the

25 polypeptide may be placed under the control of a suitable promoter which may be inducible or a constitutive promoter. Depending on the expression system, the polypeptide may be recovered from the extracellular phase, the periplasm or from the cytoplasm of the host cell.

- 30 Suitable vector systems and host cells are well-known in the art as evidenced by the vast amount of literature and materials available to the skilled person. Since the present invention also relates to the use of the nucleic acid fragments of the invention in the construction of vectors and in

host cells, the following provides a general discussion relating to such use and the particular considerations in practising this aspect of the invention.

In general, of course, prokaryotes are preferred for the
5 initial cloning of nucleic sequences of the invention and constructing the vectors useful in the invention. For example, in addition to the particular strains mentioned in the more specific disclosure below, one may mention by way of example, strains such as *E. coli* K12 strain 294 (ATCC No.
10 31446), *E. coli* B, and *E. coli* X 1776 (ATCC No. 31537). These examples are, of course, intended to be illustrative rather than limiting.

Prokaryotes are also preferred for expression, since efficient purification and protein refolding strategies are
15 available. The aforementioned strains, as well as *E. coli* W3110 (F-, lambda-, prototrophic, ATCC No. 273325), bacilli such as *Bacillus subtilis*, or other enterobacteriaceae such as *Salmonella typhimurium* or *Serratia marcesans*, and various *Pseudomonas* species may be used.

20 In general, plasmid vectors containing replicon and control sequences which are derived from species compatible with the host cell are used in connection with these hosts. The vector ordinarily carries a replication site, as well as marking sequences which are capable of providing phenotypic selection
25 in transformed cells. For example, *E. coli* is typically transformed using pBR322, a plasmid derived from an *E. coli* species (see, e.g., Bolivar et al., 1977). The pBR322 plasmid contains genes for ampicillin and tetracycline resistance and thus provides easy means for identifying transformed cells.
30 The pBR plasmid, or other microbial plasmid or phage must also contain, or be modified to contain, promoters which can be used by the microorganism for expression.

Those promoters most commonly used in recombinant DNA construction include the B-lactamase (penicillinase) and lactose

promoter systems (Chang et al., 1978; Itakura et al., 1977; Goeddel et al., 1979) and a tryptophan (trp) promoter system (Goeddel et al., 1979; EPO Appl. Publ. No. 0036776). While these are the most commonly used, other microbial promoters
5 have been discovered and utilized, and details concerning their nucleotide sequences have been published, enabling a skilled worker to ligate them functionally with plasmid vectors (Siebwenlist et al., 1980). Certain genes from prokaryotes may be expressed efficiently in *E. coli* from their own
10 promoter sequences, precluding the need for addition of another promoter by artificial means.

In addition to prokaryotes, eukaryotic microbes, such as yeast cultures may also be used. *Saccharomyces cerevisiae*, or common baker's yeast is the most commonly used among
15 eukaryotic microorganisms, although a number of other strains are commonly available. For expression in *Saccharomyces*, the plasmid YRp7, for example, is commonly used (Stinchcomb et al., 1979; Kingsman et al., 1979; Tschemper et al., 1980). This plasmid already contains the trp1 gene which provides a
20 selection marker for a mutant strain of yeast lacking the ability to grow in tryptophan for example ATCC No. 44076 or PEP4-1 (Jones, 1977). The presence of the trp1 lesion as a characteristic of the yeast host cell genome then provides an effective environment for detecting transformation by growth
25 in the absence of tryptophan.

Suitable promoting sequences in yeast vectors include the promoters for 3-phosphoglycerate kinase (Hitzman et al., 1980) or other glycolytic enzymes (Hess et al., 1968; Holland et al., 1978), such as enolase, glyceraldehyde-3-phosphate
30 dehydrogenase, hexokinase, pyruvate decarboxylase, phosphofructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triosephosphate isomerase, phosphoglucose isomerase, and glucokinase. In constructing suitable expression plasmids, the termination sequences
35 associated with these genes are also ligated into the expres-

sign vector 3' of the sequence desired to be expressed to provide polyadenylation of the mRNA and termination.

Other promoters, which have the additional advantage of transcription controlled by growth conditions are the promoter region for alcohol dehydrogenase 2, isocytochrome C, acid phosphatase, degradative enzymes associated with nitrogen metabolism, and the aforementioned glyceraldehyde-3-phosphate dehydrogenase, and enzymes responsible for maltose and galactose utilization. Any plasmid vector containing a yeast-compatible promoter, origin of replication and termination sequences is suitable.

In addition to microorganisms, cultures of cells derived from multicellular organisms may also be used as hosts. In principle, any such cell culture is workable, whether from vertebrate or invertebrate culture. However, interest has been greatest in vertebrate cells, and propagation of vertebrate in culture (tissue culture) has become a routine procedure in recent years (Tissue Culture, 1973). Examples of such useful host cell lines are VERO and HeLa cells, Chinese hamster ovary (CHO) cell lines, and W138, BHK, COS-7 293 and MDCK cell lines.

Expression vectors for such cells ordinarily include (if necessary) an origin of replication, a promoter located in front of the gene to be expressed, along with any necessary ribosome binding sites, RNA splice sites, polyadenylation site, and transcriptional terminator sequences.

For use in mammalian cells, the control functions on the expression vectors are often provided by viral material. For example, commonly used promoters are derived from polyoma, Adenovirus 2, and most frequently Simian Virus 40 (SV40). The early and late promoters of SV40 virus are particularly useful because both are obtained easily from the virus as a fragment which also contains the SV40 viral origin of replication (Fiers et al., 1978). Smaller or larger SV40 fragments

may also be used, provided there is included the approximately 250 bp sequence extending from the *HindIII* site toward the *BglII* site located in the viral origin of replication. Further, it is also possible, and often desirable, to utilize
5 promoter or control sequences normally associated with the desired gene sequence, provided such control sequences are compatible with the host cell systems.

An origin of replication may be provided either by construction of the vector to include an exogenous origin, such as
10 may be derived from SV40 or other viral (e.g., Polyoma, Adeno, VSV, BPV) or may be provided by the host cell chromosomal replication mechanism. If the vector is integrated into the host cell chromosome, the latter is often sufficient.

15 Upon production of the polypeptide monomer constructs it may be necessary to process the polypeptides further, e.g. by introducing non-proteinaceous functions in the polypeptide, by subjecting the material to suitable refolding conditions (e.g. by using the generally applicable strategies suggested
20 in WO 94/18227), or by cleaving off undesired peptide moieties of the monomer (e.g. expression enhancing peptide fragments which are undesired in the end product).

In the light of the above discussion, the methods for recombinantly producing the monomer polypeptide construct of the
25 invention are also a part of the invention, as are the vectors carrying and/or being capable of replicating the nucleic acids according to the invention in a host cell or a cell-line. According to the invention the expression vector can be e.g. a plasmid, a cosmid, a minichromosome, or a
30 phage. Especially interesting are vectors which are integrated in the host cell/cell line genome after introduction in the host.

Another part of the invention are transformed cells (useful in the above-described methods) carrying and capable of

replicating the nucleic acid fragments of the invention; the host cell can be a microorganism such as a bacterium, a yeast, or a protozoan, or a cell derived from a multicellular organism such as a fungus, an insect cell, a plant cell, or a mammalian cell. Especially interesting are cells from the bacterial species *Escherichia*, *Bacillus* and *Salmonella*, and a preferred bacterium is *E. coli*.

Yet another part of the invention relates to a stable cell line producing the polypeptide part of a monomer polypeptide construct according to the invention, and preferably the cell line carries and expresses a nucleic acid of the invention.

On the basis of the above discussions it will be clear to the skilled person that also the oligomers resulting from the complex formation between the monomer constructs of the invention are important parts of the invention. Hence the invention also pertains to an oligomer which is comprised of two monomer polypeptide constructs according to the invention which comprises at least three TTSEs, or which is comprised of three monomer polypeptide constructs according to the invention which each only contain one single TTSE.

As is explained herein and shown in the examples, the oligomers of the invention are stable at temperatures up to 70°C and it is therefore especially preferred that the oligomers of the invention are stable at temperatures above physiological ones, e.g. that the oligomers are stable in the temperature range 50-70°C.

Also a part of the invention is a method for preparing a dimeric oligomer of the invention which comprises

- admixing a monomer polypeptide construct which includes two TTSEs (construct 1) with a monomer polypeptide construct which includes only one TTSE (construct 2),

- effecting the two TTSE's of construct 1 to complex with the TTSE of construct 2 (this can be done by thermal treatment, i.e. heating to a temperature which ensures denaturation followed by subsequent cooling allowing renaturation, or this can be done by denaturing/renaturing effected by changes in the chemical environment), and
- isolating the resulting dimer and optionally subjecting the dimer to further processing (cf. the above discussion of further processing, but it should also be mentioned that the further processing could include non-covalent coupling of interesting and relevant moieties to the dimeric oligomer).

Consequently, the method for producing a trimeric oligomer is also a part of the invention and comprises the steps of

- admixing three monomer polypeptide constructs of the invention with each other,
- effecting complex formation between one TTSE of each monomer polypeptide construct, and
- isolating the resulting trimer and optionally subjecting the trimeric oligomer to further processing.

The considerations applying to complex formation and further processing mentioned above apply to this method also.

In view of the detailed discussion above of the "pick-and-mix" aspect of the invention, the invention also pertains to a kit comprising

- a first package comprising at least one container means, each at least one container means containing a monomer polypeptide construct of the invention,

- a second package comprising at least one container means,
each at least one container means in the second package
containing a monomer polypeptide construct of the inven-
tion, the second package being different from the first
5 package with respect to choice and/or number of monomer
polypeptide constructs included therein, and optionally
- a third package comprising at least one container means,
each at least one container means in the third package
containing a monomer polypeptide construct of the inven-
10 tion, the second package being different from the first
and second packages with respect to choice and/or number
of monomer polypeptide constructs included therein.

It is preferred that the at least one container means in each
package contains mutually distinct monomer polypeptide con-
15 structs, and it is especially preferred that all container
means comprised in the kit comprises mutually distinct
polypeptide constructs.

A very important aspect of the invention is the possibility
of generating a system designed especially for the individual
20 circumstances. The basic idea is that the artificial
selection of heterologous moieties and optionally active
components, and functional entities result in a unique system
as will be further disclosed in the following.

25 Using the TTSE as a vehicle for assembling monovalent scFv or
Fab antibody fragments into oligomeric and multivalent
entities offer design advantages also in terms of generating
chimaeric artificial antibodies having desirable
pharmacokinetic and pharmacodynamic properties. Small
30 derivatives like monomeric scFv fragments or bivalent
"minibodies" are rapidly cleared from the circulatory system,
whereas complete Igs stay for very much longer. Conversely,
small derivatives like scFv and minbodies exhibit better
extravasation properties. It is therefore expected that
35 antibodies of a desired specificity may be optimized for

particular diagnostic or therapeutic needs by engineering the pharmacological properties, using the TTSE as a vehicle for controlled oligomerization of e.g. scFv fragments.

One example of such engineering would be the requirements for
5 delivering a high dose of an imaging or toxin-conjugated antibody to a tumour, while ensuring as low a systemic exposure or imaging background as possible. In such case a TTSE conjugated scFv fragment could be designed to exhibit strong multivalent binding to the tumour and rapid clearance
10 of excess conjugate from circulation.

Accordingly, in af further aspect the present invention also relates to the use of a monomer polypeptide construct or to a an oligomer according to the present invention as a vehicle for assembling antibody fragments into oligomeric or
15 multivalent entities for generating chimeric artificial antibodies having preselected pharmacokinetic and/or pharmadynamic properties.

The use of specific delivery systems also play an important role in connection with the present invention in that such
20 systems may by utilized with respect to different use of the present invention both with respect to the a more general therapeutic application and with respect to gene therapy. Exampels of suitable drug delivery and targeting systems are disclosed in Nature 392 supp.(30 april 1998).

25 Accordingly, efficiency of deliverance may be further increased if the delivery system e.g a liposome is supplied with a molecular unit, an "infector or transfactor" ligand, recognized by a internalizing receptor unit specific for the target cells. For example, cells displaying endocytotic
30 receptors like members of the LDL family of receptors may be even more efficiently infected or transfected either by including a TTSE unit in the antibody containing heteorimer or in an independent TTSE unit conjugated to one or more of the domains of the Receptor Associated Protein, RAP,

(Ellgaard, L., Holtet, T.L., Nielsen, P.R., Etzerodt, M., Gliemann, J. & Thøgersen, H.C. Eur J Biochem. 1997, vol 244, 544-551) which is recognized as a ligand to all receptors in this abundant family of endocytosis-mediating receptors.

- 5 Accordingly, in a further aspect, the invention is directed to the use of a monomer polypeptide construct or to an oligomer according to the invention for targeted gene therapy involving selective delivery of the material for transfection or infection of the specific population of cells.
- 10 The ultimate perspective of such TTSE-mediated gene therapy would be the deployment of a viral vector that would find no other targets in the patient but the cells displaying the artificial receptor complex.

- In a still further aspect, the invention is directed to the
- 15 use of a monomer polypeptide construct or to a an oligomer construct according to the invention wherein the at least one heterologous moiety comprises a moiety selected from a ligand binding structure such as a receptor molecule or the ligand binding part of a receptor molecule, and wherein the gene
- 20 therapy involves the delivery of nucleic acids to the desired population of cells by use of a viral vector directed to cells displaying the artificial receptor complex corresponding to the heterologous moiety.

- In another aspect, the invention is directed to the use of a
- 25 monomer polypeptide construct or to a an oligomer construct according to the invention wherein at least one TTSE is modified with a membrane integrating or associating entity having affinity to the specific population of cells in the body relevant for the gene therapy.

- 30 Furthermore, a recent review of the imaging an therapeutic potential of a range of known antibody derivatives has been published by Paul Carter and Margaret Merchant of Genentech Inc. (Current Opinion in Biotechnology, 1997, vol 8, 449-

454). In direct continuation of their conclusions it will be apparent that oligomerisation of antibody derivatives like scFv derivatives may extend current technology in the designer-antibody field in many important aspects, some of which will be elaborated below (with reference to the Carter & Merchant review).

One of the well-known problems inherent to mouse monoclonal antibodies that have been 'humanized' by grafting of the murine antigen combining site onto a human Ig framework is that antigenicity of the chimaeric product in human patients is often difficult to suppress entirely, resulting in - sometimes life-threatening - immune reactions to the diagnostic or therapeutic humanized antibody product. The risk of such side-effects are expected to be much reduced if the designer antibody is assembled from purely human proteins or protein fragments. Since the TTSE trimerising unit described here is identical to a portion of human tetranectin that is already present in human plasma and tissue, there is good reason to expect that the TTSE will not elicit an antigenic response in a human subject if it is introduced as a component of a chimaeric product that is not otherwise antigenic in humans.

Accordingly, in one aspect, the present invention relates to the use of a monomer polypeptide construct or to a an oligomer according to the present invention as a component of a chimaeric product having low antigenicity in humans relative to formulations comprising on or more components of non-human origin.

Carter & Merchant further review present technology for radiolabelling of antibody derivatives. Again, oligomerisation using TTSEs offer more elegant solutions to problems associated with labelling, as the TTSE offers the possibility to construct one or two of the TTSE monomer units in a heterotrimeric complex to harbour the site carrying the label. Thus, in this format labelling may also be confined to

the non-antibody part of the complex, leaving the antigen-binding module entirely unmodified, and the complex may furthermore be formulated "in the field" as and when needed.

- In many receptor-mediated signal transduction pathways
- 5 signals are triggered by the clustering of receptor molecules on the cell membrane. The TTSEs therefore have important applications in the study and exploitation of receptor signalling, as ligands may be presented as oligomers by conjugation to a TTSE unit.
- 10 This also has important application in phage display technologies for discovering new ligands and new receptors as the engineering of a TTSE unit fused inline to a candidate ligand molecule will allow the display of a hetero-trimeric phage coat protein, in which only one of the monomer units is
- 15 conjugated to the phage coat protein. This may be accomplished by appropriate insertion of amber codons at the site of fusion of phage coat protein to the TTSE-ligand segment of the three-way fusion protein encoded by the recombinant phage. In appropriate E. coli cells the presence
- 20 of this amber codon will result in translation termination in the majority of read-throughs, and hence most of the fusion protein product secreted to the periplasmic compartment in the phage-infected bacterium will be soluble TTSE-ligand fusion protein, whereas a minority of the fusion protein will
- 25 also contain a phage protein module. The majority of trimers that will be generated will therefore contain, at most, one monomeric unit that will ensure integration (display) in the mature recombinant phage particle.

- A further advantage of the display technology described above
- 30 relates to the fact that it is specially useful for selection on the basis of a relatively low affinity because of the entropic benefit contribution obtained by the proximity of the tree binding moieties in confined spatial arrangement.

Accordingly, the present invention in an important aspect, also relates to protein library technology wherein the TTSE's discribed above are utilized.

The trimerisation of candidated recombinant ligands is
5 especially important as, for many receptors, the intracellular signal is induced by receptor clustering, which is only brought about if the external ligand exhibits multivalent binding to the receptor, so as to bridge two or more receptor molecules.

10 In one preferred embodiment the monomer polypeptide construct or the oligomer construct according to the invention is for targeted gene therapy involving selective delivery of the material for transfection or infection of the specific population of cells. Tthe at least one heterologous moiety may
15 comprise a moiety selected from a ligand binding structure such as a receptor molecule or the ligand binding part of a receptor molecule, and wherein the gene therapy involves the delivery of nucleic acids to the desired population of cells by use of a viral vector directed to cells displaying the
20 artificial receptor complex corresponding to the heterologous moiety.

As mentioned above, it is an important aspect of the invention that the monomer polypeptide construct and/or the oligomer may be used as a component of a chimaeric product
25 having low antigenicity in humans. As the construc is of human origin it is believed that the antigenicity in humans is low relative to formulations comprising on or more components of non-human origin.

One primary use of a monomer polypeptide construct or a an
30 oligomer according to the invention is for delivering an imaging or toxin-conjugated antibody to a target such as a tumor, or use as a vehicle delivering an substance to a target cell or tissue, as a vehicle for assembling antibody fragments into oligomeric or multivalent entities for

generating chimeric artificial antibodies having preselected pharmacokinetic and/or pharmacodynamic properties.

The substance in question being one or more selected from the group of heterologous moieties as well a pharmaceutical. Also
5 a labelled construct wherein the label is coupled to one or to of the TTSE monomer units is within the scope of the invention.

As explained in detail previously, an important and surprising use of the monomer polypeptide construct or the
10 oligomer according to the present invention is for protein library technology, such as phage display technology. The present invention also relates to any polynucleotide molecule such as a RNA, DNA or PNA as well as any vector encoding one or more TTSE.

15 A further use according to invention includes preparation and use of a pharmaceutical composition comprising the TTSE construct and optionally a pharmaceutically acceptable excipient. The composition may be administered by a route selected from the group consisting of the intravenous route,
20 the intraarterial route, the transmembrane route of the buccal, anal, vaginal or conjunctival tissue, the intranasal route, the pulmonary route, the transdermal route, the intramuscular route, subcutaneous route, intratechal route, inoculation into tissue such as a tumour, or by an implant.

25 The the monomer polypeptide construct or the oligomer is in a preferred embodiment comprised in a liposome.

It is obvious from the disclosure of the present invention that the treating or preventing of a disease may by a further aspect comprising administering to the subject in need
30 thereof an effective amount of a pharmaceutical composition referred to above.

In one aspect of the various possibilities according to the present invention concerning how the human gene therapy is targeted, includes the case wherein at least one TTSE is modified with a membrane integrating or associating entity
5 having affinity to the specific population of cells in the body relevant for the gene therapy.

As used in the conventional pharmaceutical field the present invention includes a method wherein the monomer polypeptide construct or the oligomer is administered by a route selected
10 from the group consisting of the intravenous route, the intraarterial route, the transmembrane route of the buccal, anal og vaginal tissue, intranasal route, the pulmonary route, the transdermal route, intramuscular, subcutaneous, intratechal, the buccal, inoculation into tissue such as a
15 tumour, or by an implant.

Finally, the present invention is also relating to the field of dianosing as the skilled person would easily recognise, that the TTSE disclosen in the present inventiion may also refer to a method for diagnosis comprising a construct
20 comprising the monomer polypeptide construct or the oligomer, together with a diagnosing component coupled thereon.

EXAMPLE 1

*Design and construction of the pTH6trip E. coli expression vectors for the production of trimerised chimeric fusion
25 proteins.*

The plasmid clone pT7H6FXTN123 (Example 2) was used as template for amplification in two Polymerase Chain Reactions (PCR) (Saiki et al., 1988) with the primer pairs trip-N (SEQ ID NO: 1) and trip-Ca (SEQ ID NO: 2) and trip-N (SEQ ID NO:
30 1) and trip-Cb (SEQ ID NO: 3), respectively. The amplified DNA fragments, tripa, comprising nucleotide sequences encoding an IQGR cleavage site for the restriction protease FX_a

(SEQ ID NO: 4) followed by two sites for the restriction nucleases BglII and KpnI, the nucleotide sequence encoding the tetranectin polypeptide sequence for Glu 1 to Lys 52 (SEQ ID NO: 5) followed by recognition sites for the three restriction endonucleases BamHI, HindIII, and EcoRI, respectively, and tripb, comprising nucleotide sequences encoding an IQGR cleavage site for the restriction protease FX_a (SEQ ID NO: 4) followed by two sites for the restriction nucleases BglII and KpnI, the nucleotide sequence encoding the tetranectin polypeptide sequence for Glu 1 to Val 49 (SEQ ID NO: 6) followed by recognition sites for the three restriction endonucleases BamHI, HindIII, and EcoRI, respectively, were subcloned into the plasmid pT7H6 (Christensen et al., 1991), yielding pTtripa and pTtripb, respectively (Figs. 3 and 4).

15 EXAMPLE 2

Tetranectin, localisation of the trimerising structural element and stability of the triple alpha helical coiled coil.

The cDNA encoding the reading frame corresponding to the mature tetranectin single chain (SEQ ID NO: 7) was cloned by specific amplification in a Polymerase Chain Reaction (PCR) (Saiki et al., 1988) of the nucleotide sequences from amino acid residue Glu1 to Val181 using 1st strand oligo-dT primed cDNA synthesized from total human placental RNA as template. Primers used in the PCR were SEQ ID NO: 8 and SEQ ID NO: 9. RNA extraction and cDNA synthesis were performed using standard procedures. The amplified reading frame encoding the monomer subunit of tetranectin was at the 5'-end, via the PCR-reaction, linked to nucleotide sequences encoding the amino acid sequence SEQ ID NO: 10 which constitute an IEGR cleavage site for the bovine restriction protease FX_a (Nagai, and Thøgersen, 1987). A glycine residue was, due to the specific design of the 5'-PCR primer (SEQ. ID NO. 8), inserted between the C-terminal arginine residue of the FX_a cleavage site (SEQ ID NO. 10) and the tetranectin Glu1-resi-

due. The amplified DNA fragment was subcloned into the *E. coli* expression vector pT₇H₆ (Christensen et al., 1991) producing the plasmid pT₇H₆FX-TN123 expressing the tetranectin monomer H6FXTN123 (SEQ ID NO: 25) and into pT₇CIIH₆, which is a derivative of pT₇H₆, where the amino-terminal 32 amino acid residues of the lambda CII protein (SEQ ID NO. 11) are inserted 5' of the six histidine residues (SEQ ID NO. 12) as outlined in Fig. 5, yielding pT₇CIIH₆FX-TN123 expressing the tetranectin fusion protein CIIH6FXTN123 (SEQ ID NO: 24).

The amino acid sequence of the expressed proteins are shown in Fig. 6 (in SEQ ID NO: 7 is given the amino acid sequence of the mature tetranectin protein). Furthermore three additional derivatives of tetranectin were constructed (Fig. 8): H6FXTN12 comprising the tetranectin amino acid residues Glu1 to Val49 (SEQ ID NO: 6), H6FXTN23 comprising the tetranectin amino acid residues Val17 to Val181 (SEQ ID NO: 7), and H6FXTN3 (SEQ ID NO: 30) comprising the tetranectin amino acid residues Ala45 to Val181 (SEQ ID NO: 7). These three tetranectin derivatives were constructed by specific amplification in a PCR using pT₇H₆FX-TN123 as template and the primer-pairs SEQ ID NO: 8 and SEQ ID NO: 13, SEQ ID NO: 14 and SEQ ID NO: 9, and SEQ ID NO: 15 and SEQ ID NO: 9, respectively. The amplified DNA fragments were subcloned into the *E. coli* expression vector pT₇H₆ producing the plasmids pT₇H₆FX-TN12, pT₇H₆FX-TN23, and pT₇H₆FX-TN3, respectively (Fig. 7).

To prepare recombinant tetranectin and its derivatives, each of the plasmids pT₇H₆FX-TN123, pT₇CIIH₆FX-TN123, pT₇H₆FX-TN12, pT₇H₆FX-TN23, and pT₇H₆FX-TN3 were grown in medium scale (4 x 1 litre; 2xTY medium, 5 mM MgSO₄ and 100 µg ampicillin) in *E. coli* BL21 cells, as described by Studier et al. (1990). Exponentially growing cultures at 37°C were at OD₆₀₀ 0.8 infected with bacteriophage lambda CE6 at a multiplicity of approximately 5. Cultures were grown at 37°C for another three hours and the cells harvested by centrifugation.

Cells were resuspended in 150 ml of 0.5 M NaCl, 10 mM Tris-HCl pH 8, and 1 mM EDTA pH 8. Phenol (100 ml adjusted to

pH 8) was added and the mixture sonicated to extract the total protein. Protein was precipitated from the phenol phase by 2.5 volumes of ethanol and centrifugation.

The protein pellet was dissolved in a buffer containing 6M
5 guanidinium chloride, 50 mM Tris-HCl pH 8 and 0.1 M dithio-
erythriol. Following gel filtration on Sephadex G-25 (Pharma-
cia, Sweden) into 8 M Urea, 1 M NaCl, 50 mM Tris-HCl pH 8 and
10 mM 2-mercaptoethanol, the crude protein preparation was
applied to a Ni^{2+} activated NTA-agarose column (Ni^{2+} NTA-aga-
10 rose, 75 ml pre-washed with 8 M urea, 1 M NaCl, 50 mM
Tris-HCl pH 8, and 10 mM 2-mercaptoethanol) for purification
(Hochuli et al., 1988) and refolding of the fusion proteins,
H6FXTN123, CIIH6FXTN123, H6FXTN12, H6FXTN23, and H6FXTN3.

For this study we chose to prepare our own Ni^{2+} NTA-agarose
15 matrix. A carbodiimide coupling of the N-(5-amino-1-carboxy-
pentyl) iminodiacetic acid metal ligand (synthesis route as
described by Döbeli & Hochuli (EP-A-0 253 303)) to a rigid
agarose matrix (Sephacrose CL-6B, Pharmacia, Sweden) was
performed:

20 8 g of N-(5-amino-1-carboxypentyl)iminodiacetic acid from the
synthesis procedure in 50 ml was adjusted to pH 10 by addi-
tion of 29 g of Na_2CO_3 (10 H_2O) and added to a stirred suspen-
sion of activated Sepharose CL-6B in 1 M Na_2CO_3 . Reaction was
allowed overnight. The Sepharose CL-6B (initially 100 ml
25 suspension) was activated after removal of water by acetone
with 7 g of 1,1'-carbonyldiimidazol under stirring for 15 to
30 min. Upon activation the Sepharose CL-6B was washed with
acetone followed by water and 1 M Na_2CO_3 .

The NTA-agarose matrix was loaded into a column and "charged"
30 with Ni^{2+} by slowly passing through 5 column volumes of a 10%
 NiSO_4 solution. The amount of Ni^{2+} on the NTA-agarose matrix,
prepared by this procedure, has been determined to 14 μmol
per ml matrix. After charging the Ni^{2+} NTA-agarose column was
washed with two column volumes of water, one column volume of

1 M Tris-HCl pH 8 and two column volumes of loading buffer before stirred mixing of the Ni^{2+} NTA-agarose matrix with the crude protein extracts in a breaker for 15 to 30 min. All buffers prepared for liquid chromatography were degassed under vacuum prior to addition of reductant and/or use.

The Ni^{2+} NTA-agarose matrix - crude extract mixture was packed in standard glass columns for liquid chromatography (internal diameter: 2.6 cm) to a volume of approximately 40 ml. The columns were washed with 200 ml of 8 M urea, 1 M NaCl, 50 mM Tris-HCl pH 8, and 10 mM 2-mercaptoethanol (Buffer I) and 100 ml 6 M guanidinium chloride, 50 mM Tris-HCl pH 8 and 10 mM 2-mercaptoethanol (Buffer II) and the adsorbed tetranectin derived fusion proteins H6FXTN123, H6CIIFXTN123, H6FXTN23, and H6FXTN3 refolded using the cyclic refolding procedure as described (Thøgersen et al., WO 94/18227).

The fusion protein H6FXTN12 was refolded by removing the guanidinium chloride and 2-mercaptoethanol of buffer II in a gradient over 5 column volumes into 50 mM Tris-HCl pH 8 and 0.5 M NaCl. After completion of the refolding procedures the tetranectin derived fusion proteins were eluted from the Ni^{2+} NTA-agarose columns with a buffer containing 0.5 M NaCl, 50 mM Tris-HCl, 25 mM EDTA pH 8. The tetranectin fusion proteins H6FXTN123, H6FXTN23, and H6FXTN3 were cleaved with FX_a at 4°C overnight in a molar ratio of 1:300. After FX_a cleavage the protein samples were concentrated 10 fold by ultrafiltration on YM10 membranes (Amicon). After ten times dilution of the protein sample with 2 mM CaCl_2 , the recombinant tetranectin derivatives TN123, TN23, and TN3 were isolated by ion-exchange chromatography on Q-Sepharose (Pharmacia, Sweden) in a linear gradient over 10 column volumes from 10 mM Tris-HCl pH 8, 2 mM CaCl_2 to 10 mM Tris-HCl pH 8, 2 mM CaCl_2 , and 0.5 M NaCl. After elution from the Ni^{2+} NTA-agarose columns the fusion proteins H6CIIFXTN123 and H6FXTN12 were likewise concentrated 10 fold by ultrafiltration on YM10 membranes and gelfiltrated into buffer containing 25 mM Tris-HCl pH 8, 25 mM NaCl, and 2 mM CaCl_2 , before purification of

correctly folded monomer by ion-exchange chromatography on Q-Sepharose as described.

Recombinant full length tetranectin (TN123) produced by these procedures have been analyzed with respect to binding to plasminogen kringle 4 and immobilised fucoidan, expression of antigenic sites, and localization of disulphide bridges. In all criteria tested the produced TN123 behaved identically to isolated naturally human tetranectin (data not shown). Furthermore TN123 and TN3 have been crystallized (Kastrup et al., 1996) and the structure has also been determined, all of which bear evidence that a single unique and biologically active folded product had indeed been produced.

Analytical gelfiltration analysis of rTN proteins.

Analytical gelfiltration of the recombinant tetranectin derivatives TN123, TN3, and TN23 (Fig. 9) were performed on a Superose 12 HR 10/30 column (Pharmacia, Sweden) with a total volume of 25 ml in 100 mM NaCl and 50 mM Tris-HCl pH 8 and a flow rate of 0.2 ml/min. The K_{av} value is defined by, $K_{av} = (V_e - V_o) / (V_c - V_o)$.

The gelfiltration analysis of TN123 and TN23 show that both proteins are exclusively found as trimers in solution (K_{av} values of 0.27 and 0.29, respectively), whereas TN3 appeared monomeric (K_{av} :0.41).

Chemical cross-linking of tetranectin and derivatives

The recombinant tetranectin derivatives TN123, TN3, and TN23, together with the fusion proteins CIIH6FXTN123 and H6FXTN12 or mixtures of these derivatives at 1 mg/ml concentrations in cross-linking buffer (0.1 M Sodium borate, pH 9.1) were incubated with dimethylsuberimidate (DMSI, Sigma). 10 μ l aliquots of protein solution were incubated with 1 μ l aliquots of DMSI stock solution (20 mg/ml in cross-linking buffer) for 30 minutes at 25°C before addition of 2 μ l quenching

buffer (3 M Tris-HCl, pH 9). Subunit exchange between pre-formed homo-oligomers was induced by subjecting protein mixtures to heat shock treatment. Five μ l aliquots of each protein solution (1 mg/ml stocks) were mixed at 0°C in standard polypropylene microcentrifuge tubes, transferred to a water bath at 70°C for the time spans indicated, and then further incubated for 15 minutes at 25°C before reaction with DMSI.

- 10 Prior to analysis by SDS-PAGE (12% gels) of the cross-linked products the reaction samples were boiled in the presence of SDS and mercaptoethanol.

Cross-linking analysis of TN123 and the fusion protein CIIH6FXTN123 showed that no detectable subunit exchange between pre-formed homo-oligomers in a mixture of TN123 and CIIH6FXTN123 was found after 16 hours at room temperature (Fig. 10). Subunit exchange could be induced by incubating the protein mixture at 70°C for 15 seconds or longer before cooling to room temperature and addition of DMSI. SDS-PAGE analysis showed the presence of four trimer bands above 95 kDa (corresponding to two homo-trimers and two hetero-trimers) and three dimer bands (corresponding to two homo-dimers and one hetero-dimer) in the gel between 43 and 55 kDa, in a relative abundance in agreement with random association of monomer subunits into trimers after subunit exchange. It should be noted, that molecular weight markers have only been included on the SDS-PAGE gels for crude calibration and orientation of the gels.

The trimeric organization of tetranectin was further corroborated by cross-linking studies of the proteins H6FXTN12, TN23, and TN3 and mixtures between them (Fig. 11). The tetranectin derivative TN3, containing only the CRD, could not be cross-linked even at high protein concentrations and did not interfere with the cross-linking of rTN123. Likewise, the derivative TN23, containing exon 2 and the CRD, appeared monomeric after cross-linking and was found not to interfere

with trimerisation of TN123 during subunit exchange. Dimeric TN23 molecules found at low abundance in the sample probably reflects contaminating misfolded disulphide bridged dimers. The fusion protein H6FXTN12 formed homo-trimers upon cross-linking and generated hetero-trimers with TN123 after subunit exchange. Because of the difference in size of full length tetranectin (TN123) and H6FXTN12 the possible nine protein bands resulting from chemical cross-linking are: The four trimers [(TN123)₃, (TN123)₂(H6FXTN12), (TN123)(H6FXTN12)₂, and (H6FXTN12)₃] at approx. 95 kDa, 50 kDa, 37 kDa, and 20 kDa, respectively; the three dimers [(TN123)₂, (TN123)(H6FXTN12), and (H6FXTN12)₂] at approx. 45 kDa, 30 kDa, and 15 kDa, respectively; and the two monomers TN123 at 23 kDa and H6FXTN12 at 9 kDa.

Taken together, the gel filtration and the cross-linking analysis of the tetranectin derivatives show that tetranectin, like the collectin group of C-type lectins, is a trimeric molecule and that amino acid residues directly shown to be involved in trimerisation of the tetranectin monomer are located in exon 2 of the protein (Val17 - Val49). Furthermore subunit exchange between the trimeric molecules could only be observed after heat denaturation. Amino acid residues Glu1 to Asp16 of tetranectin are critical to chemical cross-linking with DMSI and more important appear to stabilize the trimeric molecule because the cross-linking analysis of the mixture TN123 and TN23 showed no decrease in TN123 formation after heat denaturation and possible subunit exchange (Fig. 11). The stability of the tetranectin trimer was corroborated by a cross-linking analysis with DMSI at different temperatures. Fifteen μ l TN123 at 0.3 mg/ml concentration was pre-incubated 10 min. at either 37°C, 50°C, 60°C, or 70°C before addition of 2 μ l DMSI (20 mg/ml). The reaction was allowed to proceed for 15 min. before reaction was quenched with 5 μ l of 3M Tris-HCl pH 9.1 and the reaction mixtures allowed to cool to room temperature. SDS-PAGE analysis of reduced samples (Fig. 12) showed, that trimers are readily detectable even at 60°C, although a competing pattern

of cross-linking specimens increases at increasing temperatures. The appearance of other cross-linking specimens is probably due to the unfolding of the CRD. The stability of the tetranectin trimerising structural element is further
5 analyzed using a designed chimeric protein in Example 3.

EXAMPLE 3

Design and construction of the recombinant chimeric protein TRIPB-UB - the tetranectin trimerising structural element and ubiquitin.

- 10 A plasmid clone, pLCMHF/UB, generously provided by Dr. O. Wiborg harbouring a human ubiquitin cDNA insert (SEQ ID: 16) was used as template and SEQ ID NO: 17 together with SEQ ID NO: 18 were used for amplification in a Polymerase Chain Reaction (PCR) (Saiki et al., 1988) of the nucleotide
15 sequence encoding amino acid residue Ile1 to Gly76 of human ubiquitin (SEQ ID: 19). The amplified DNA fragment was after digestion with the restriction endonucleases BamHI and HindIII ligated into the BamHI and HindIII sites of pTtripb (Example 1) yielding pTtripb-UB (Fig. 13) using standard
20 procedures.

To prepare the chimeric fusion protein H6FXtripb-UB (Fig. 14, SEQ ID NO: 31) the plasmid pTtripb-UB was grown in medium scale (4 x 1 litre; 2xTY medium, 5 mM MgSO₄ and 100 µg ampicillin) in *E. coli* BL21 cells, as described by Studier et
25 al. (1990). Exponentially growing cultures at 37°C were at OD₆₀₀ 0.8 infected with bacteriophage lambda CE6 at a multiplicity of approximately 5. Cultures were grown at 37°C for another three hours and the cells harvested by centrifugation. Cells were resuspended in 150 ml of 0.5 M
30 NaCl, 10 mM Tris-HCl pH 8, and 1 mM EDTA pH 8. Phenol (100 ml adjusted to pH 8) was added and the mixture sonicated to extract the total protein. Protein was precipitated from the phenol phase by 2.5 volumes of ethanol and centrifugation. The protein pellet was dissolved in a buffer containing 6 M

guanidinium chloride, 50 mM Tris-HCl pH 8 and 0.1 M dithioerythriol. Following gel filtration on Sephadex G-25 (Pharmacia, Sweden) into 8 M Urea, 1 M NaCl, 50 mM Tris-HCl pH 8 and 10 mM 2-mercaptoethanol, the crude protein preparation was applied to a Ni^{2+} activated NTA-agarose column for purification (Hochuli et al., 1988) and refolding of the fusion protein H6FXtripb-UB.

Synthesis and charging of the Ni^{2+} activated NTA-agarose matrix is described in Example 2. All buffers for liquid chromatography were degassed prior to use. The fusion protein H6FXtripb-UB was refolded by removing the urea and 2-mercaptoethanol from buffer II in a gradient over 5 column volumes into 50 mM Tris-HCl pH 8 and 0.5 M NaCl. After completion of the refolding procedure the H6FXtripb-UB fusion protein was eluted from the Ni^{2+} NTA-agarose columns with a buffer containing 0.5 M NaCl, 50 mM Tris-HCl, 25 mM EDTA pH 8 and gel filtrated on a Sephadex G50 column (Pharmacia) into 0.1 M Sodium borate pH 9 buffer for chemical cross-linking analysis with DMSI.

The cross-linking analysis experiment was designed both to analyze the oligomeric status of the chimeric fusion protein and the thermal stability of the presumed fusion protein trimer as described in Example 2: Samples of 15 μl H6FXtripb-UB fusion protein, at approximately 1.0 mg/ml concentration, were pre-incubated 10 min. at either 37°C, 50°C, 60°C, or 70°C before addition of 2 μl DMSI (20 mg/ml). The reactions were allowed to proceed for 15 min. before quenching by addition of 5 μl of 3 M Tris-HCl pH9.1 and the reaction mixtures were allowed to cool to room temperature. SDS-PAGE analysis of reduced samples (Fig. 12) showed, (1) that the fusion protein H6FXtripb-UB is a trimer in solution (monomer at 17 kDa, dimer at 35 kDa, and trimer at 43 kDa) and (2) that a substantial amount of trimer molecules is present even at 70°C. The appearance of other larger cross-linking products is probably due to cross-linking of trimers via the ubiquitin part of the fusion protein.

EXAMPLE 4

Design and construction of trimerised and hexamerized CEA6 scFv antibodies scFv(CEA6)-TRIPB, TRIPB-scFv(CEA6) and scFv(CEA6)-TRIPB-scFv(CEA6).

- 5 A plasmid clone, pUC19MCH/CEA6, generously provided by Dr. Kevin Pritchard, Cambridge Antibody Technology Ltd., Melbourne, UK, harbouring a nucleotide sequence (SEQ ID: 20) encoding the CEA6 antibody in single-chain Fv (scFv) format, followed in sequence by a "myc tag" (which is a general
10 purification/detection handle), was used as template in Polymerase Chain Reactions (PCR) (Saiki et al., 1988) in which the nucleotide sequence encoding the scFv + myc tag was amplified using the primer pairs (SEQ ID: 21 and SEQ ID: 22) and (SEQ ID: 21 and SEQ ID: 23) to generate PCR fragments "A"
15 and "B".

- PCR fragment "A" was treated with restriction enzymes BamHI and KpnI and the resulting fragment was inserted into BglIII/KpnI cut pTripb (Example 1) to obtain the vector pTH6FXscFv(CEA6)-tripb (Fig. 15) encoding the H6FXscFv(CEA6)-
20 TRIPB fusion protein (Fig. 16). PCR fragment "B" was treated with restriction enzymes BamHI and HindIII and the resulting fragment was inserted into BamHI and HindIII cut pTripb (Example 1) to obtain the vector pTH6FXtripb-scFv(CEA6) (Fig. 17) encoding the H6FXTRIPB-scFv(CEA6) fusion protein (Fig. 18,
25 SEQ ID NO: 33) using standard procedures.

- To generate the expression vector pTH6FXscFv(CEA6)-tripb-scFv(CEA6) (Fig. 19) encoding the H6FXscFv(CEA6)-TRIPB-scFv(CEA6) fusion protein (Fig. 20, SEQ ID NO: 34) the insert in the vector pTH6FXtripb-scFv(CEA6) was excised using re-
30 striction enzymes BamHI and HindIII and inserted into the vector pTH6FXscFv(CEA6)-tripb, which had been treated with restriction enzymes BamHI and HindIII.

To prepare the chimeric fusion proteins H6FXscFv(CEA6)-TRIPB (SEQ ID NO: 32), H6FXTRIPB-scFv(CEA6) (SEQ ID NO: 33) and H6FXscFv(CEA6)-TRIPB-scFv(CEA6) (SEQ ID NO: 34) the plasmids pTH6FXscFv(CEA6)-TRIPB, pTH6FXtripb-scFv(CEA6) and

5 pTH6FXscFv(CEA6)-tripb-scFv(CEA6) were grown in small scale (1 litre; 2xTY medium, 5 mM MgSO₄ and 100 µg ampicillin) in E. coli BL21 cells, as described by Studier et al. (1990). Exponentially growing cultures at 37°C were at OD₆₀₀ 0.8 infected with bacteriophage lambda CE6 at a multiplicity of

10 approximately 5. Cultures were grown at 37°C for another three hours and the cells harvested by centrifugation. Cells were resuspended in 50 ml of 0.5 M NaCl, 50 mM Tris-HCl pH 8, and 1 mM EDTA pH 8. Phenol (50 ml adjusted to pH 8) was added to each and the mixtures were sonicated to extract total

15 protein. After clarification by centrifugation (25 minutes at 10.000 g) crude protein fractions were precipitated from the phenol phases by addition of 2.5 volumes of ethanol and centrifugation. Protein pellets were dissolved in a buffer (15-25 ml) containing 6 M guanidinium chloride, 50 mM

20 Tris-HCl pH 8 and 0.1 M dithioerythriol. Following gel filtration on Sephadex G-25 (Pharmacia, Sweden) into 8 M Urea, 1 M NaCl, 50 mM Tris-HCl pH 8 and 10 mM 2-mercaptoethanol, the crude protein preparations were applied to Ni²⁺ activated NTA-agarose columns (75 ml column volume) for purification

25 (Hochuli et al., 1988). Washing buffer (6 M guanidine-HCl, 50 mM Tris-HCl pH 8 and 10 mM 2-mercaptoethanol) was then flowed through the columns until stable baselines were obtained. Virtually pure fusion proteins could then be eluted by applying a pH gradient to each column (1000 ml gradient in 8 M

30 urea and 10 mM 2-mercaptoethanol obtained by linear (per volume) mixing of solutions containing 50 mM sodium di-hydrogenphosphate (pH 5 buffer) and 50 mM di-sodium hydrogenphosphate (pH 8 buffer).

In preparation for in vitro refolding by the method of Thøgersen et al. (WO 94/18227) 20 mg of each purified fusion

35 protein were mixed in suspensions in refolding "buffer B" (described below) with aliquots of suspensions of Ni²⁺ acti-

vated NTA-agarose matrix sufficient to generate columns of about 75 ml packed bed volume. Each fusion protein was then subjected to the iterative refolding procedure as described for plasminogen kringle 4 in the Thøgersen et al. patent application (WO 94/18227), except that refolding of the scFv containing fusion proteins was carried out at 10°C using a buffer containing 0.5 M NaCl, 50 mM Tris-HCl pH 8, 2 mM glutathione and 0.2 mM oxidized glutathione as "buffer A" and a buffer containing 8 M urea, 1 M NaCl, 50 mM Tris-HCl pH 8 and 2 mM glutathione as "buffer B".

After completion of the refolding procedure each column was washed with 300 ml buffer containing 0.5 M NaCl and 50 mM Tris-HCl pH 8 to wash away glutathione. The refolded fraction of each protein was then eluted from the NTA-agarose matrix by addition of 20 mM EDTA to the elution buffer. After addition of solid urea to achieve a final concentration of about 8 M to each protein sample and dilution or dialysis to reduce NaCl concentrations to below 5 mM, final purification of each correctly folded fusion protein product was then accomplished by ion exchange chromatography (S-Sepharose, Pharmacia, 1,6 (i.d.) by 90 centimeter column in a buffer containing 8 M urea, 5 mM Tris-HCl (from 1 M stock solution at pH 8) and 25 mM sodium acetate (from 1 M stock solution at pH 5), eluted at 2 ml/min). After dialysis against aqueous buffers (e.g. phosphate buffered saline) each pure and correctly refolded fusion protein was recovered in yields of 2-6 mg per litre of culture grown. Each protein may be shown by analytical gel filtration, chemical cross-linking analysis, by in vitro affinity measurements and by in vivo efficacy to form a stable homotrimeric molecular complex: The oligomeric status of the H6FXtripb-scFv-(CEA6) fusion protein was analyzed by chemical cross-linking analysis with DMSI: In parallel experiments, samples of H6FXtripb-scFv-(CEA6) at 0.34 mg/ml and TN123 at 0.28 mg/ml in 0.1 M Sodium borate were incubated at room temperature with increasing amounts (0 - 40 µg in 12 µl in total) of DMSI for 30 min. Reactions were quenched by addition of 5 µl 3M Tris-HCl pH 9 and the samples analyzed by

SDS-PAGE under reducing conditions (Fig. 21). Like tetranectin, the H6FXtripb-scFV-(CEA6) fusion protein, of approximately 38 kDa, is hereby shown to be a trimer in solution.

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SEQUENCE LISTING

(1) GENERAL INFORMATION

(i) APPLICANT

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- (C) CITY: Mundelstrup
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- (E) COUNTRY: Denmark
- (F) POSTAL CODE: 8381

(ii) TITLE OF THE INVENTION: Trimerising module

(iii) NUMBER OF SEQUENCES: 34

(iv) COMPUTER-READABLE FORM:

- (A) MEDIUM TYPE: Diskette
- (B) COMPUTER: IBM Compatible
- (C) OPERATING SYSTEM: DOS
- (D) SOFTWARE: FastSEQ for Windows Version 2.0

(v) CURRENT APPLICATION DATA:

- (A) APPLICATION NUMBER:

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 47 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

CCTGATCAAT CCAGGGAAGA TCTCCTGGTA CCGAGCCACC AACCCAG

47

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 33 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

CCAAGCTTAT TAGGATCCCG TCTGCAGGGC CTG

33

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 40 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GCGAAGCTTA TTAGGATCCC TTCAGGGAGA CCGTCTGCAG

40

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 6 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: None

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Gly Ser Ile Gln Gly Arg
1 5

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 52 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: None

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Glu Pro Pro Thr Gln Lys Pro Lys Lys Ile Val Asn Ala Lys Lys Asp
1 5 10 15
Val Val Asn Thr Lys Met Phe Glu Glu Leu Lys Ser Arg Leu Asp Thr
20 25 30
Leu Ala Gln Glu Val Ala Leu Leu Lys Glu Gln Gln Ala Leu Gln Thr
35 40 45
Val Ser Leu Lys
50

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 49 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: None

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Glu Pro Pro Thr Gln Lys Pro Lys Lys Ile Val Asn Ala Lys Lys Asp
1 5 10 15

Val Val Asn Thr Lys Met Phe Glu Glu Leu Lys Ser Arg Leu Asp Thr
 20 25 30
 Leu Ala Gln Glu Val Ala Leu Leu Lys Glu Gln Gln Ala Leu Gln Thr
 35 40 45
 Val

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 181 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: None

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Glu Pro Pro Thr Gln Lys Pro Lys Lys Ile Val Asn Ala Lys Lys Asp
 1 5 10 15
 Val Val Asn Thr Lys Met Phe Glu Glu Leu Lys Ser Arg Leu Asp Thr
 20 25 30
 Leu Ala Gln Glu Val Ala Leu Leu Lys Glu Gln Gln Ala Leu Gln Thr
 35 40 45
 Val Cys Leu Lys Gly Thr Lys Val His Met Lys Cys Phe Leu Ala Phe
 50 55 60
 Thr Gln Thr Lys Thr Phe His Glu Ala Ser Glu Asp Cys Ile Ser Arg
 65 70 75 80
 Gly Gly Thr Leu Ser Thr Pro Gln Thr Gly Ser Glu Asn Asp Ala Leu
 85 90 95
 Tyr Glu Tyr Leu Arg Gln Ser Val Gly Asn Glu Ala Glu Ile Trp Leu
 100 105 110
 Gly Leu Asn Asp Met Ala Ala Glu Gly Thr Trp Val Asp Met Thr Gly
 115 120 125
 Ala Arg Ile Ala Tyr Lys Asn Trp Glu Thr Glu Ile Thr Ala Gln Pro
 130 135 140
 Asp Gly Gly Lys Thr Glu Asn Cys Ala Val Leu Ser Gly Ala Ala Asn
 145 150 155 160
 Gly Lys Trp Phe Asp Lys Arg Cys Arg Asp Gln Leu Pro Tyr Ile Cys
 165 170 175
 Gln Phe Gly Ile Val
 180

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 39 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

CCTGGATCCA TCGAGGGTAG GGGCGAGCCA CCAACCCAG

39

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 25 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

CCGAAGCTTA CACGATCCCG AACTG

25

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 6 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: None

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

Gly Ser Ile Glu Gly Arg
 1 5

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 32 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: None

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

Met Val Arg Ala Asn Lys Arg Asn Glu Ala Leu Arg Ile Glu Ser Ala
 1 5 10 15
 Leu Leu Asn Lys Ile Ala Met Leu Gly Thr Glu Lys Thr Ala Glu Gly
 20 25 30

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 10 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: None

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

Gly Ser His His His His His Gly Ser
 1 5 10

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 25 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

CCGAAGCTTA GACCGTCTGC AGGGC

25

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 39 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

GGCGGATCCA TCCAGGGTAG GGTGTGAAC ACAAAGATG

39

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 36 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

CCTGGATCCA TCGAGGGTAG GGCCCTGCAG ACGGTC

36

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 227 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

ATGCAGATCT TTGTGAAGAC CCTCACTGGC AAAACCATCA CCCTTGAGGT CGAGCCCAGT	60
GACACCATTG AGAATGTCAA AGCCAAAATT CAAGACAAGG AGGGTATCCC ACCTGACCGC	120
ACCGTCTGAT ATTTGCCGGC AAACAGCTGG AAGATGGACG TACTTTGTCT GACTACAATA	180
TTCAAAAGGA GTCTACTCTT CATCTTGTGT TGAGACTTCG TGGTGGT	227

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 27 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

TGCTGATCAC AGATCTTTGT GAAGACC

27

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 39 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

CGCAAGCTTG CATGCTTAGG ATCCACCACG AAGTCTCAA

39

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 76 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: None

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

Met	Gln	Ile	Phe	Val	Lys	Thr	Leu	Thr	Gly	Lys	Thr	Ile	Thr	Leu	Glu
1				5					10					15	
Val	Glu	Pro	Ser	Asp	Thr	Ile	Glu	Asn	Val	Lys	Ala	Lys	Ile	Gln	Asp
			20					25					30		
Lys	Glu	Gly	Ile	Pro	Pro	Asp	Gln	Gln	Arg	Leu	Ile	Phe	Ala	Gly	Lys
		35					40					45			
Gln	Leu	Glu	Asp	Gly	Arg	Thr	Leu	Ser	Asp	Tyr	Asn	Ile	Gln	Lys	Glu
	50					55				60					
Ser	Thr	Leu	His	Leu	Val	Leu	Arg	Leu	Arg	Gly	Gly				
65					70					75					

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 786 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

CAGGTTTCAGC TGCAGCAGTC AGGGGCTGAG GTGAAGAAGC CTGGGTCCTC GGTGAAGGTC

60

TCCTGCAAGG	CTTCTGGAGG	CACCTTCAGC	AACTCTCCTA	TCAACTGGCT	GCGACAGGCC	120
CCCGGACAAG	GGCTTGAGTG	GATGGGAAGT	ATCATCCCTT	CCTTTGGTAC	AGCAAACACTAC	180
GCTCAGAAGT	TCCAGGGCAG	ACTCACGATT	ACGCGGACG	AATCCACGAG	CACAGCCTAC	240
ATGGAGCTGA	GCAGCCTGAG	ATCTGAGGAC	ACGGCCGTGT	ATTACTGTGC	GGGGCGGAGC	300
CACAACTACG	AACTCTACTA	TTACTACATG	GACGTCTGGG	GCCAGGGGAC	AATGGTCACC	360
GTCTCGAGTG	GTGGAGGCGG	TTCAGGCGGA	GGTGGCAGCG	GCGGTGGCGG	ATCGGACATC	420
CAGATGACCC	AGTCTCCTTC	CACCCTGTCT	GCATCTATTG	GAGACAGAGT	CACCATCACC	480
TGCCGGGCCA	GTGAGGGTAT	TTATCACTGG	TGGCCTGGT	ATCAGCAGAA	GCCAGGGAAA	540
GCCCTAAAC	TCCTGATCTA	TAAGGCCTCT	AGTTTAGCCA	GTGGGGCCCC	ATCAAGGTTC	600
AGCGGCAGTG	GATCTGGGAC	AGATTTCACT	CTCACCATCA	GCAGCCTGCA	GCCTGATGAT	660
TTTGCAACTT	ATTACTGCCA	ACAATATAGT	AATTATCCGC	TCACTTTCGG	CGGAGGGACC	720
AAGCTGGAGA	TCAAACGTGC	GGCCGCAGAA	CAAAAACCTCA	TCTCAGAAGA	GGATCTGAAT	780
GGGGCC						786

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 25 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

GGTGGATCCC AGGTTACAGCT GCAGC

25

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 25 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

GCCGGTACCG GCCCATTCATCA GATCC

25

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 26 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

TCCAAGCTTA GGCCCCATTC AGATCC

26

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 228 amino acids
- (B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: None

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

```

Met Val Arg Ala Asn Lys Arg Asn Glu Ala Leu Arg Ile Glu Ser Ala
 1           5           10           15
Leu Leu Asn Lys Ile Ala Met Leu Gly Thr Glu Lys Thr Ala Glu Gly
      20           25           30
Gly Ser His His His His His Gly Ser Ile Glu Gly Arg Gly Glu
      35           40           45
Pro Pro Thr Gln Lys Pro Lys Lys Ile Val Asn Ala Lys Lys Asp Val
 50           55           60
Val Asn Thr Lys Met Phe Glu Glu Leu Lys Ser Arg Leu Asp Thr Leu
65           70           75           80
Ala Gln Glu Val Ala Leu Leu Lys Glu Gln Gln Ala Leu Gln Thr Val
      85           90           95
Cys Leu Lys Gly Thr Lys Val His Met Lys Cys Phe Leu Ala Phe Thr
      100          105          110
Gln Thr Lys Thr Phe His Glu Ala Ser Glu Asp Cys Ile Ser Arg Gly
      115          120          125
Gly Thr Leu Ser Thr Pro Gln Thr Gly Ser Glu Asn Asp Ala Leu Tyr
      130          135          140
Glu Tyr Leu Arg Gln Ser Val Gly Asn Glu Ala Glu Ile Trp Leu Gly
      145          150          155          160
Leu Asn Asp Met Ala Ala Glu Gly Thr Trp Val Asp Met Thr Gly Ala
      165          170          175
Arg Ile Ala Tyr Lys Asn Trp Glu Thr Glu Ile Thr Ala Gln Pro Asp
      180          185          190
Gly Gly Lys Thr Glu Asn Cys Ala Val Leu Ser Gly Ala Ala Asn Gly
      195          200          205
Lys Trp Phe Asp Lys Arg Cys Arg Asp Gln Leu Pro Tyr Ile Cys Gln
      210          215          220
Phe Gly Ile Val
225

```

(2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 197 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: None

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

```

Met Gly Ser His His His His His His Gly Ser Ile Glu Gly Arg Gly
 1           5           10           15
Glu Pro Pro Thr Gln Lys Pro Lys Lys Ile Val Asn Ala Lys Lys Asp
      20           25           30
Val Val Asn Thr Lys Met Phe Glu Glu Leu Lys Ser Arg Leu Asp Thr
      35           40           45
Leu Ala Gln Glu Val Ala Leu Leu Lys Glu Gln Gln Ala Leu Gln Thr
      50           55           60

```

```

Val Cys Leu Lys Gly Thr Lys Val His Met Lys Cys Phe Leu Ala Phe
65              70              75              80
Thr Gln Thr Lys Thr Phe His Glu Ala Ser Glu Asp Cys Ile Ser Arg
85              90              95
Gly Gly Thr Leu Ser Thr Pro Gln Thr Gly Ser Glu Asn Asp Ala Leu
100            105            110
Tyr Glu Tyr Leu Arg Gln Ser Val Gly Asn Glu Ala Glu Ile Trp Leu
115            120            125
Gly Leu Asn Asp Met Ala Ala Glu Gly Thr Trp Val Asp Met Thr Gly
130            135            140
Ala Arg Ile Ala Tyr Lys Asn Trp Glu Thr Glu Ile Thr Ala Gln Pro
145            150            155            160
Asp Gly Gly Lys Thr Glu Asn Cys Ala Val Leu Ser Gly Ala Ala Asn
165            170            175
Gly Lys Trp Phe Asp Lys Arg Cys Arg Asp Gln Leu Pro Tyr Ile Cys
180            185            190
Gln Phe Gly Ile Val
195

```

(2) INFORMATION FOR SEQ ID NO:26:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 65 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: None

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

```

Met Gly Ser His His His His His Gly Ser Ile Glu Gly Arg Gly
1              5              10              15
Glu Pro Pro Thr Gln Lys Pro Lys Lys Ile Val Asn Ala Lys Lys Asp
20            25            30
Val Val Asn Thr Lys Met Phe Glu Glu Leu Lys Ser Arg Leu Asp Thr
35            40            45
Leu Ala Gln Glu Val Ala Leu Leu Lys Glu Gln Gln Ala Leu Gln Thr
50            55            60
Val
65

```

(2) INFORMATION FOR SEQ ID NO:27:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 180 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: None

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

```

Met Gly Ser His His His His His Gly Ser Ile Gln Gly Arg Val
1              5              10              15
Val Asn Thr Lys Met Phe Glu Glu Leu Lys Ser Arg Leu Asp Thr Leu
20            25            30

```


Ala	Gln	Glu	Val	Ala	Leu	Leu	Lys	Glu	Gln	Gln	Ala	Leu	Gln	Thr	Val
35				40				45							
Cys	Leu	Lys	Gly	Thr	Lys	Val	His	Met	Lys	Cys	Phe	Leu	Ala	Phe	Thr
50				55				60							
Gln	Thr	Lys	Thr	Phe	His	Glu	Ala	Ser	Glu	Asp	Cys	Ile	Ser	Arg	Gly
65				70				75				80			
Gly	Thr	Leu	Ser	Thr	Pro	Gln	Thr	Gly	Ser	Glu	Asn	Asp	Ala	Leu	Tyr
				85				90				95			
Glu	Tyr	Leu	Arg	Gln	Ser	Val	Gly	Asn	Glu	Ala	Glu	Ile	Trp	Leu	Gly
100								105				110			
Leu	Asn	Asp	Met	Ala	Ala	Glu	Gly	Thr	Trp	Val	Asp	Met	Thr	Gly	Ala
115				120				125							
Arg	Ile	Ala	Tyr	Lys	Asn	Trp	Glu	Thr	Glu	Ile	Thr	Ala	Gln	Pro	Asp
130				135				140							
Gly	Gly	Lys	Thr	Glu	Asn	Cys	Ala	Val	Leu	Ser	Gly	Ala	Ala	Asn	Gly
145				150				155				160			
Lys	Trp	Phe	Asp	Lys	Arg	Cys	Arg	Asp	Gln	Leu	Pro	Tyr	Ile	Cys	Gln
				165				170				175			
Phe	Gly	Ile	Val												
180															

(2) INFORMATION FOR SEQ ID NO:28:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 73 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: None

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

[illegible]

(2) INFORMATION FOR SEQ ID NO:29:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 69 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: None

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

Met Gly Ser His His His His His His Gly Ser Ile Gln Gly Arg Ser
1 5 10 15

```

Pro Gly Thr Glu Pro Pro Thr Gln Lys Pro Lys Lys Ile Val Asn Ala
      20                      25                      30
Lys Lys Asp Val Val Asn Thr Lys Met Phe Glu Glu Leu Lys Ser Arg
      35                      40                      45
Leu Asp Thr Leu Ala Gln Glu Val Ala Leu Leu Lys Glu Gln Gln Ala
      50                      55                      60
Leu Gln Thr Gly Ser
65

```

(2) INFORMATION FOR SEQ ID NO:30:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 152 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: None

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

```

Met Gly Ser His His His His His His Gly Ser Ile Glu Gly Arg Ala
 1                      5                      10                      15
Leu Gln Thr Val Cys Leu Lys Gly Thr Lys Val His Met Lys Cys Phe
      20                      25                      30
Leu Ala Phe Thr Gln Thr Lys Thr Phe His Glu Ala Ser Glu Asp Cys
      35                      40                      45
Ile Ser Arg Gly Gly Thr Leu Ser Thr Pro Gln Thr Gly Ser Glu Asn
      50                      55                      60
Asp Ala Leu Tyr Glu Tyr Leu Arg Gln Ser Val Gly Asn Glu Ala Glu
65                      70                      75                      80
Ile Trp Leu Gly Leu Asn Asp Met Ala Ala Glu Gly Thr Trp Val Asp
      85                      90                      95
Met Thr Gly Ala Arg Ile Ala Tyr Lys Asn Trp Glu Thr Glu Ile Thr
      100                      105                      110
Ala Gln Pro Asp Gly Gly Lys Thr Glu Asn Cys Ala Val Leu Ser Gly
      115                      120                      125
Ala Ala Asn Gly Lys Trp Phe Asp Lys Arg Cys Arg Asp Gln Leu Pro
      130                      135                      140
Tyr Ile Cys Gln Phe Gly Ile Val
145                      150

```

(2) INFORMATION FOR SEQ ID NO:31:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 145 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: None

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

```

Met Gly Ser His His His His His His Gly Ser Ile Gln Gly Arg Ser
 1                      5                      10                      15
Pro Gly Thr Glu Pro Pro Thr Gln Lys Pro Lys Lys Ile Val Asn Ala
      20                      25                      30

```

```

Lys Lys Asp Val Val Asn Thr Lys Met Phe Glu Glu Leu Lys Ser Arg
   35                               40                               45
Leu Asp Thr Leu Ala Gln Glu Val Ala Leu Leu Lys Glu Gln Gln Ala
   50                               55                               60
Leu Gln Thr Gly Ser Gln Ile Phe Val Lys Thr Leu Thr Gly Lys Thr
   65                               70                               75                               80
Ile Thr Leu Glu Val Glu Pro Ser Asp Thr Ile Glu Asn Val Lys Ala
                               85                               90                               95
Lys Ile Gln Asp Lys Glu Gly Ile Pro Pro Asp Gln Gln Arg Leu Ile
                               100                               105                               110
Phe Ala Gly Lys Gln Leu Glu Asp Gly Arg Thr Leu Ser Asp Tyr Asn
                               115                               120                               125
Ile Gln Lys Glu Ser Thr Leu His Leu Val Leu Arg Leu Arg Gly Gly
   130                               135                               140
Ser
145

```

(2) INFORMATION FOR SEQ ID NO:32:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 330 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: None

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:

```

Met Gly Ser His His His His His His Gly Ser Ile Gln Gly Arg Ser
  1                               5                               10                               15
Gln Val Gln Leu Gln Gln Ser Gly Ala Glu Val Lys Lys Pro Gly Ser
  20                               25                               30
Ser Val Lys Val Ser Cys Lys Ala Ser Gly Gly Thr Phe Ser Asn Ser
  35                               40                               45
Pro Ile Asn Trp Leu Arg Gln Ala Pro Gly Gln Gly Leu Glu Trp Met
  50                               55                               60
Gly Ser Ile Ile Pro Ser Phe Gly Thr Ala Asn Tyr Ala Gln Lys Phe
  65                               70                               75                               80
Gln Gly Arg Leu Thr Ile Thr Ala Asp Glu Ser Thr Ser Thr Ala Tyr
                               85                               90                               95
Met Glu Leu Ser Ser Leu Arg Ser Glu Asp Thr Ala Val Tyr Tyr Cys
                               100                               105                               110
Ala Gly Arg Ser His Asn Tyr Glu Leu Tyr Tyr Tyr Tyr Met Asp Val
                               115                               120                               125
Trp Gly Gln Gly Thr Met Val Thr Val Ser Ser Gly Gly Gly Gly Ser
  130                               135                               140
Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser Asp Ile Gln Met Thr Gln
  145                               150                               155                               160
Ser Pro Ser Thr Leu Ser Ala Ser Ile Gly Asp Arg Val Thr Ile Thr
                               165                               170                               175
Cys Arg Ala Ser Glu Gly Ile Tyr His Trp Leu Ala Trp Tyr Gln Gln
                               180                               185                               190
Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile Tyr Lys Ala Ser Ser Leu
                               195                               200                               205
Ala Ser Gly Ala Pro Ser Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp
  210                               215                               220

```

```

Phe Thr Leu Thr Ile Ser Ser Leu Gln Pro Asp Asp Phe Ala Thr Tyr
225                230                235                240
Tyr Cys Gln Gln Tyr Ser Asn Tyr Pro Leu Thr Phe Gly Gly Gly Thr
                245                250                255
Lys Leu Glu Ile Lys Arg Ala Ala Ala Glu Gln Lys Leu Ile Ser Glu
                260                265                270
Glu Asp Leu Asn Gly Ala Gly Thr Glu Pro Pro Thr Gln Lys Pro Lys
                275                280                285
Lys Ile Val Asn Ala Lys Lys Asp Val Val Asn Thr Lys Met Phe Glu
                290                295                300
Glu Leu Lys Ser Arg Leu Asp Thr Leu Ala Gln Glu Val Ala Leu Leu
305                310                315                320
Lys Glu Gln Gln Ala Leu Gln Thr Gly Ser
                325                330

```

(2) INFORMATION FOR SEQ ID NO:33:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 331 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: None

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:33:

```

Met Gly Ser His His His His His Gly Ser Ile Gln Gly Arg Ser
 1                5                10                15
Pro Gly Thr Glu Pro Pro Thr Gln Lys Pro Lys Lys Ile Val Asn Ala
                20                25                30
Lys Lys Asp Val Val Asn Thr Lys Met Phe Glu Glu Leu Lys Ser Arg
 35                40                45
Leu Asp Thr Leu Ala Gln Glu Val Ala Leu Leu Lys Glu Gln Gln Ala
 50                55                60
Leu Gln Thr Gly Ser Gln Val Gln Leu Gln Gln Ser Gly Ala Glu Val
 65                70                75                80
Lys Lys Pro Gly Ser Ser Val Lys Val Ser Cys Lys Ala Ser Gly Gly
                85                90                95
Thr Phe Ser Asn Ser Pro Ile Asn Trp Leu Arg Gln Ala Pro Gly Gln
                100                105                110
Gly Leu Glu Trp Met Gly Ser Ile Ile Pro Ser Phe Gly Thr Ala Asn
                115                120                125
Tyr Ala Gln Lys Phe Gln Gly Arg Leu Thr Ile Thr Ala Asp Glu Ser
                130                135                140
Thr Ser Thr Ala Tyr Met Glu Leu Ser Ser Leu Arg Ser Glu Asp Thr
 145                150                155                160
Ala Val Tyr Tyr Cys Ala Gly Arg Ser His Asn Tyr Glu Leu Tyr Tyr
                165                170                175
Tyr Tyr Met Asp Val Trp Gly Gln Gly Thr Met Val Thr Val Ser Ser
                180                185                190
Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser Asp
                195                200                205
Ile Gln Met Thr Gln Ser Pro Ser Thr Leu Ser Ala Ser Ile Gly Asp
                210                215                220
Arg Val Thr Ile Thr Cys Arg Ala Ser Glu Gly Ile Tyr His Trp Leu
225                230                235                240

```

```

Ala Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile Tyr
      245                      250                      255
Lys Ala Ser Ser Leu Ala Ser Gly Ala Pro Ser Arg Phe Ser Gly Ser
      260                      265                      270
Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu Gln Pro Asp
      275                      280                      285
Asp Phe Ala Thr Tyr Tyr Cys Gln Gln Tyr Ser Asn Tyr Pro Leu Thr
      290                      295                      300
Phe Gly Gly Gly Thr Lys Leu Glu Ile Lys Arg Ala Ala Ala Glu Gln
305                      310                      315                      320
Lys Leu Ile Ser Glu Asp Leu Asn Gly Ala
      325                      330

```

(2) INFORMATION FOR SEQ ID NO:34:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 592 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: None

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:34:

```

Met Gly Ser His His His His His His Gly Ser Ile Gln Gly Arg Ser
 1           5           10           15
Gln Val Gln Leu Gln Gln Ser Gly Ala Glu Val Lys Lys Pro Gly Ser
      20           25           30
Ser Val Lys Val Ser Cys Lys Ala Ser Gly Gly Thr Phe Ser Asn Ser
      35           40           45
Pro Ile Asn Trp Leu Arg Gln Ala Pro Gly Gln Gly Leu Glu Trp Met
50           55           60
Gly Ser Ile Ile Pro Ser Phe Gly Thr Ala Asn Tyr Ala Gln Lys Phe
65           70           75           80
Gln Gly Arg Leu Thr Ile Thr Ala Asp Glu Ser Thr Ser Thr Ala Tyr
      85           90           95
Met Glu Leu Ser Ser Leu Arg Ser Glu Asp Thr Ala Val Tyr Tyr Cys
      100          105          110
Ala Gly Arg Ser His Asn Tyr Glu Leu Tyr Tyr Tyr Tyr Met Asp Val
      115          120          125
Trp Gly Gln Gly Thr Met Val Thr Val Ser Ser Gly Gly Gly Gly Ser
130          135          140
Gly Gly Gly Gly Ser Gly Gly Gly Ser Asp Ile Gln Met Thr Gln
145          150          155          160
Ser Pro Ser Thr Leu Ser Ala Ser Ile Gly Asp Arg Val Thr Ile Thr
      165          170          175
Cys Arg Ala Ser Glu Gly Ile Tyr His Trp Leu Ala Trp Tyr Gln Gln
      180          185          190
Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile Tyr Lys Ala Ser Ser Leu
      195          200          205
Ala Ser Gly Ala Pro Ser Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp
210          215          220
Phe Thr Leu Thr Ile Ser Ser Leu Gln Pro Asp Asp Phe Ala Thr Tyr
225          230          235          240
Tyr Cys Gln Gln Tyr Ser Asn Tyr Pro Leu Thr Phe Gly Gly Gly Thr
      245          250          255

```

Lys Leu Glu Ile Lys Arg Ala Ala Ala Glu Gln Lys Leu Ile Ser Glu
 260 265 270
 Glu Asp Leu Asn Gly Ala Gly Thr Glu Pro Pro Thr Gln Lys Pro Lys
 275 280 285
 Lys Ile Val Asn Ala Lys Lys Asp Val Val Asn Thr Lys Met Phe Glu
 290 295 300
 Glu Leu Lys Ser Arg Leu Asp Thr Leu Ala Gln Glu Val Ala Leu Leu
 305 310 315 320
 Lys Glu Gln Gln Ala Leu Gln Thr Gly Ser Gln Val Gln Leu Gln Gln
 325 330 335
 Ser Gly Ala Glu Val Lys Lys Pro Gly Ser Ser Val Lys Val Ser Cys
 340 345 350
 Lys Ala Ser Gly Gly Thr Phe Ser Asn Ser Pro Ile Asn Trp Leu Arg
 355 360 365
 Gln Ala Pro Gly Gln Gly Leu Glu Trp Met Gly Ser Ile Ile Pro Ser
 370 375 380
 Phe Gly Thr Ala Asn Tyr Ala Gln Lys Phe Gln Gly Arg Leu Thr Ile
 385 390 395 400
 Thr Ala Asp Glu Ser Thr Ser Thr Ala Tyr Met Glu Leu Ser Ser Leu
 405 410 415
 Arg Ser Glu Asp Thr Ala Val Tyr Tyr Cys Ala Gly Arg Ser His Asn
 420 425 430
 Tyr Glu Leu Tyr Tyr Tyr Tyr Met Asp Val Trp Gly Gln Gly Thr Met
 435 440 445
 Val Thr Val Ser Ser Gly Gly Gly Ser Gly Gly Gly Gly Ser Gly
 450 455 460
 Gly Gly Gly Ser Asp Ile Gln Met Thr Gln Ser Pro Ser Thr Leu Ser
 465 470 475 480
 Ala Ser Ile Gly Asp Arg Val Thr Ile Thr Cys Arg Ala Ser Glu Gly
 485 490 495
 Ile Tyr His Trp Leu Ala Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro
 500 505 510
 Lys Leu Leu Ile Tyr Lys Ala Ser Ser Leu Ala Ser Gly Ala Pro Ser
 515 520 525
 Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser
 530 535 540
 Ser Leu Gln Pro Asp Asp Phe Ala Thr Tyr Tyr Cys Gln Gln Tyr Ser
 545 550 555 560
 Asn Tyr Pro Leu Thr Phe Gly Gly Gly Thr Lys Leu Glu Ile Lys Arg
 565 570 575
 Ala Ala Ala Glu Gln Lys Leu Ile Ser Glu Glu Asp Leu Asn Gly Ala
 580 585 590

CLAIMS

1. A monomer polypeptide construct comprising at least one tetranectin trimerising structural element (TTSE) which is covalently linked to at least one heterologous moiety, said
5 TTSE being capable of forming a stable complex with two other TTSEs, with the proviso that the heterologous moiety is different from any of the fusion proteins CIIH6FXTN123, H6FXTN123, H6FXTN12, H6FXTN23, the sequences of which are shown in SEQ ID NOS: 24-27.
- 10 2. A monomer polypeptide construct comprising at least one tetranectin trimerising structural element (TTSE) which is covalently linked to at least one heterologous moiety, said TTSE being capable of forming a stable complex with two other TTSEs, said at least one heterologous moiety being one which
15 does not exclusively facilitate expression and/or purification of the monomer polypeptide construct.
3. A monomer polypeptide construct according to claim 1 or 2, wherein the stable complex includes a triple alpha helical coiled coil.
- 20 4. A monomer polypeptide construct according to any of the preceding claims, wherein the heterologous moiety is selected from the group consisting of a ligand binding structure; a toxin; a detectable label; an *in situ* activatable substance; an enzyme; a radioactive moiety; a cytokine; a non-protein-
25 aceous polymer such as a polymeric alkaloid, a polyalcohol, a polysaccharide, a lipid and a polyamine; a photo cross-linking agent; and a group facilitating conjugation of the monomer polypeptide construct to a target.
- 30 5. A monomer polypeptide construct according to any of the preceding claims, which comprises 2 TTSEs which are covalently linked by a spacer moiety which allows both of the 2 TTSEs to take part in complex formation with a third TTSE not being part of the monomer polypeptide construct.

6. A monomer polypeptide construct according to claim 5, wherein the spacer moiety has a length and a conformation which favours complex formation involving both of the two TTSEs which are covalently linked by the spacer moiety.
- 5 7. A monomer polypeptide construct according to claim 5 or 6, wherein the spacer moiety is a polypeptide fragment.
8. A monomer polypeptide construct according to any of claims 1-4, which comprises one single TTSE.
9. A monomer polypeptide construct according to any of the
10 preceding claims, wherein the TTSE is derived from human tetranectin, murine tetranectin, C-type lectin of bovine cartilage, or C-type lectin of shark cartilage.
10. A monomer polypeptide construct according to claim 9, wherein the TTSE comprises a polypeptide sequence which has
15 at least 68% sequence identity with the consensus sequence shown in Fig. 2.
11. A monomer polypeptide construct according to claim 10, wherein the sequence identity with the consensus sequence is at least 75%, such as at least 81%, at least 87%, or at least
20 92%.
12. A monomer polypeptide construct according to any of claims 9-11, wherein at least one amino acid residue selected from the group consisting of amino acid residue nos. 6, 21, 22, 24, 25, 27, 28, 31, 32, 35, 39, 41, 42, is/are substituted by any non-helix breaking amino acid residue, the amino
25 acid residue numbering referring to amino acid residues in SEQ ID NO: 7.
13. A monomer polypeptide construct according to any of the preceding claims, wherein the at least one TTSE comprises a
30 repeated heptad having the formula a-b-c-d-e-f-g (N to C),

wherein a majority of the amino acids residues a and d are hydrophobic amino acids.

14. A monomer polypeptide construct according to claim 13, wherein heptad is repeated 3 times and wherein the last
5 occurrence of the heptad has a glutamine residue corresponding to residues a and d.

15. A monomer polypeptide construct according to any of the preceding claims, wherein the at least one heterologous moiety is covalently linked to the TTSE via a peptide bond to
10 the N- or C-terminus of the TTSE peptide chain, via a peptide bond to a side chain in the TTSE, via a bond to a cysteine residue, or when more than one heterologous moiety, combinations of these locations.

16. A monomer polypeptide construct according to any of the
15 preceding claims which lacks any free amino and/or carboxy groups.

17. A monomer polypeptide construct according to any of the preceding claims which lacks a substantial part of the N-terminal region of tetranectin which is encoded by exon 1.

20 18. A monomer polypeptide construct according to any of the preceding claims comprising two heterologous moieties which are linked via peptide bonds to the N- and C-terminus, respectively.

19. A monomer polypeptide construct according to any of the
25 preceding claims which is constructed so as to disfavour formation of complexes between identical TTSEs.

20. An oligomer which is comprised of two monomer polypeptide constructs according to any of claims 1-19, and which comprises at three TTSE's or a multiplicity of three TTSE's, or
30 which is comprised of three monomer polypeptide constructs according to any of claims 1-4 or 8-19.

21. An oligomer according to claim 20 which is stable in the temperature range 50-70°C.
22. An oligomer according to claim 20 or 21, which comprises at least one heterologous moiety which is positioned N-terminally to a TTSE and at least one heterologous moiety which is positioned C-terminally to a TTSE.
23. An oligomer according to claim 22, wherein the at least one heterologous moiety which is positioned N-terminally to a TTSE and the at least one heterologous moiety which is positioned C-terminally to a TTSE are part of the same monomeric polypeptide construct.
24. An oligomer according to claim 22, wherein the at least one heterologous moiety which is positioned N-terminally to a TTSE and the at least one heterologous moiety which is positioned C-terminally to a TTSE are part of two separate monomeric polypeptide constructs.
25. An oligomer according to any of claims 20-24, wherein each monomer polypeptide construct is designed so as to disfavour formation of trimers including two monomer polypeptide constructs having identical TTSEs.
26. A method of preparing a monomer polypeptide construct according to any of claims 1-19, the method comprising
- isolating the monomer polypeptide construct from a culture comprising a host cell which carries and expresses a nucleic acid fragment which encodes the monomer polypeptide construct, or
 - synthesizing, by means of chemical peptide synthesis, the monomer polypeptide construct and subsequently isolating the monomer polypeptide construct from the reaction mixture, or

- preparing a TTSE in a culture comprising a host cell which carries and expresses a nucleic acid fragment which encodes the TTSE, subsequently linking covalently at least one heterologous moiety to the TTSE, and thereafter isolating the resulting monomer polypeptide construct, or
- synthesizing, by means of chemical peptide synthesis, a TTSE, subsequently linking covalently at least one heterologous moiety to the TTSE, and thereafter isolating the resulting monomer polypeptide construct from the reaction mixture,

and optionally subjecting the monomer polypeptide construct to further processing.

27. A method for preparing a dimeric oligomer according to claim 20 which comprises

- admixing a monomer polypeptide construct according to any of claims 1-19 which includes two TTSEs (construct 1) with a monomer polypeptide construct according to any of claims 1-4 or 8-19 which includes only one TTSE (construct 2),
- effecting the two TTSE's of construct 1 to complex with the TTSE of construct 2, and
- isolating the resulting dimer and optionally subjecting the dimer to further processing.

28. A method for preparing a trimeric oligomer according to claim 20 which comprises

- admixing three monomer polypeptide constructs according to any of claims 1-19 with each other,
- effecting complex formation between one TTSE of each monomer polypeptide construct, and

- isolating the resulting trimer and optionally subjecting the trimeric oligomer to further processing.

29. A kit comprising

- 5 - a first package comprising at least one container means, each at least one container means containing a monomer polypeptide construct according to any of claims 1-19,
- 10 - a second package comprising at least one container means, each at least one container means in the second package containing a monomer polypeptide construct according to any of claims 1-19, the second package being different from the first package with respect to choice and/or number of monomer polypeptide constructs included therein, and optionally
- 15 - a third package comprising at least one container means, each at least one container means in the third package containing a monomer polypeptide construct according to any of claims 1-19, the second package being different from the first and second packages with respect to choice and/or number of monomer polypeptide constructs included
- 20 therein.

30. A kit according to claim 29, wherein the at least one container means in each package contains mutually distinct monomer polypeptide constructs.

25 31. A kit according to claim 29 or 30, wherein all container means comprised in the kit comprises mutually distinct polypeptide constructs.

32. A nucleic acid fragment in isolated form which encodes a TTSE as defined in any of claims 1-19 or which encodes the polypeptide part of a monomer polypeptide construct according to any of claims 1-19, with the proviso that the nucleic acid

30 fragment is different from one that encodes native members of

the tetranectin family, and that the nucleic acid fragment is different from one that encodes any of the fusion proteins CIIH6FXTN123, H6FXTN123, H6FXTN12, H6FXTN23, the sequences of which are shown in SEQ ID NOS: 24-27.

5 33. A replicable vector which comprises a nucleic acid fragment according to claim 32.

34. A transformed host cell, which comprises a nucleic acid fragment according to claim 32 or a replicable vector according to claim 32.

10 35. Use of a monomer polypeptide construct according to any of claims 1-19 or to a an oligomer construct according to any of claims 20-25 for targeted gene therapy involving selective delivery of the material for transfection or infection of the specific population of cells.

15 36. The use according to claim 35 wherein the at least one heterologous moiety comprises a moiety selected from a ligand binding structure such as a receptor molecule or the ligand binding part of a receptor molecule, and wherein the gene therapy involves the delivery of nucleic acids to the desired
20 population of cells by use of a viral vector directed to cells displaying the artificial receptor complex corresponding to the heterologous moiety.

37. The use of a monomer polypeptide construct according to any of claims 1-19 or to a an oligomer according to any of
25 claims 20-25 as a component of a chimaeric product having low antigenicity in humans relative to formulations comprising on or more components of non-human origin.

38. The use of a monomer polypeptide construct according to any of claims 1-19 or to a an oligomer according to any of
30 claims 20-25 as a vehicle for assembling antibody fragments into oligomeric or multivalent entities for generating

chimeric artificial antibodies having preselected pharmacokinetic and/or pharmacodynamic properties.

39. The use of a monomer polypeptide construct according to any of claims 1-19 or to a an oligomer according to any of
5 claims 20-25 for delivering an imaging or toxin-conjugated antibody to a tumor.

40. The use of a monomer polypeptide construct according to any of claims 1-19 or to a oligomer according to any of claims 20-25 as a vehicle delivering an substance to a target
10 cell or tissue.

41. The use of a monomer polypeptide construct according to any of claims 1-19 or to a oligomer according to any of claims 20-25 for a labelled construct wherein the label is coupled to one or to of the TTSE monomer units.

15 42. The use of a monomer polypeptide construct according to any of claims 1-19 or to a oligomer according to any of claims 20-25 for protein library technology, such as phage display technology.

43. The use according to claim 42 comprising a poly
20 nucleotide molecule encoding one or more TTSE.

44. The use according to claim 43 comprising a vector encoding one or more TTSE.

45. The use of a monomer polypeptide construct according to any of claims 1-19 or to a oligomer according to any of
25 claims 20-25 for the preparation of a pharmaceutical composition.

46. The use according to any to claim 45, wherein the pharmaceutical composition further comprises a pharmaceutically acceptable excipient.

47. The use according to claim 45 or 46 wherein the pharmaceutical composition is administered by a route selected from the group consisting of the intravenous route, the intraarterial route, the transmembrane route of the buccal, anal, vaginal or conjunctival tissue, the intranasal route, the pulmonary route, the transdermal route, the intramuscular route, subcutaneous route, intratechal route, inoculation into tissue such as a tumour, or by an implant.
48. The use according to any of claims 35 to 47 wherein the monomer polypeptide construct according to any of claims 1-19 or the oligomer according to any of claims 20-25 is comprised in a liposome
49. A method for treating or preventing of a disease comprising administering to the subject in need thereof an effective amount of a pharmaceutical composition as defined in any of claims 45 and 46.
50. A method for treating or preventing a disease comprising administering to the subject in need thereof an effective amount of a relevant pharmaceutical coupled to a monomer polypeptide construct according to any of claims 1-19 or to an oligomer according to any of claims 20-25.
51. A method for targeted gene therapy comprising use of a monomer polypeptide construct according to any of claims 1-19 or to an oligomer according to any of claims 20-25.
52. A method of human gene therapy comprising use of a monomer polypeptide construct according to any of claims 1-19 or to an oligomer according to any of claims 20-25 wherein at least one TTSE is modified with a membrane integrating or associating entity having affinity to the specific population of cells in the body relevant for the gene therapy.
53. A method according to any of claims 49 to 52

wherein the monomer polypeptide construct according to any of claims 1-19 or the oligomer according to any of claims 20-25 is administered by a route selected from the group consisting of the intravenous route, the intraarterial route, the transmembrane route of the buccal, anal og vaginal tissue, intranasal route, the pulmonary route, the transdermal route, intramuscular, subcutaneous, intratechal, the buccal, inoculation into tissue such as a tumour, or by an implant.

54. A method for prevention and/or treating a disease, comprising administering to a mammal in need thereof a prophylactically or therapeutically effective amount of a construct comprising the monomer polypeptide construct according to any of claims 1-19 or the oligomer according to any of claims 20-25.

55. A method for diagnosis comprising a construct comprising the monomer polypeptide construct according to any of claims 1-19 or the oligomer according to any of claims 20-25 together with a diagnosing component coupled thereon.

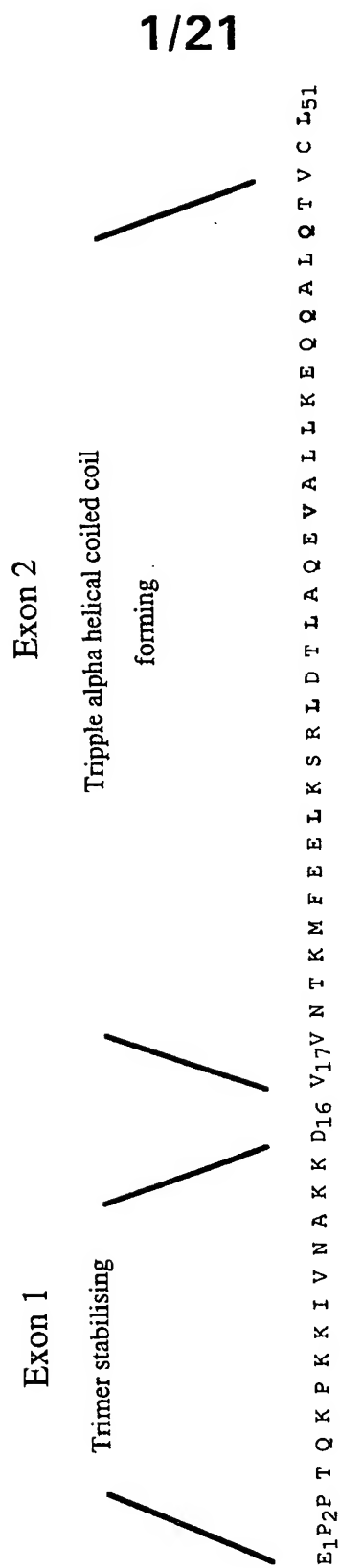


Fig. 1

2/21

Position	d	e	f	g	a	b	c	d	e	f	g	a	b	c	d	e	f	g	a	b	c	d	e	f	g	a										
Human tetranectin	V	V	N	T	K	M	F	E	E	L	K	S	R	L	D	T	L	A	Q	E	V	A	L	L	K	E	Q	Q	A	L	Q	T	V	C	L	K
Murine tetranectin	L	V	S	S	K	M	F	E	E	L	K	N	R	M	D	V	L	A	Q	E	V	A	L	L	K	E	K	Q	A	L	Q	T	V	C	L	K
Bovine cart. protein	R	R	V	K	E	K	D	G	D	L	K	T	Q	V	E	K	L	W	R	E	V	N	A	L	K	E	M	Q	A	L	Q	T	V	C	L	R
Shark cart. protein	S	K	S	G	K	G	K	D	D	L	R	N	E	I	D	K	L	W	R	E	V	N	S	L	K	E	M	Q	A	L	Q	T	V	C	L	K
Consensus	L										h	y	L						E	V					L	K	E	Q	A	L	Q	T	V	C	L	

Fig. 2

3/21

tripa

G S I Q G R S P G T E P P T T V S L K G S *
 GATCAATCCAGGGAAGATCTCTGGTACCGAGCCACCAACC ----- ACGTCTCCCTGAAGGGATCCTAA
 Bgl II Kpn II Bam HI

tripb

G S I Q G R S P G T E P P T A L Q T G S *
 GATCAATCCAGGGAAGATCTCTGGTACCGAGCCACCAACC ----- GCCCTGCAGACGGGATCCTAA
 Bgl II Kpn II Bam HI

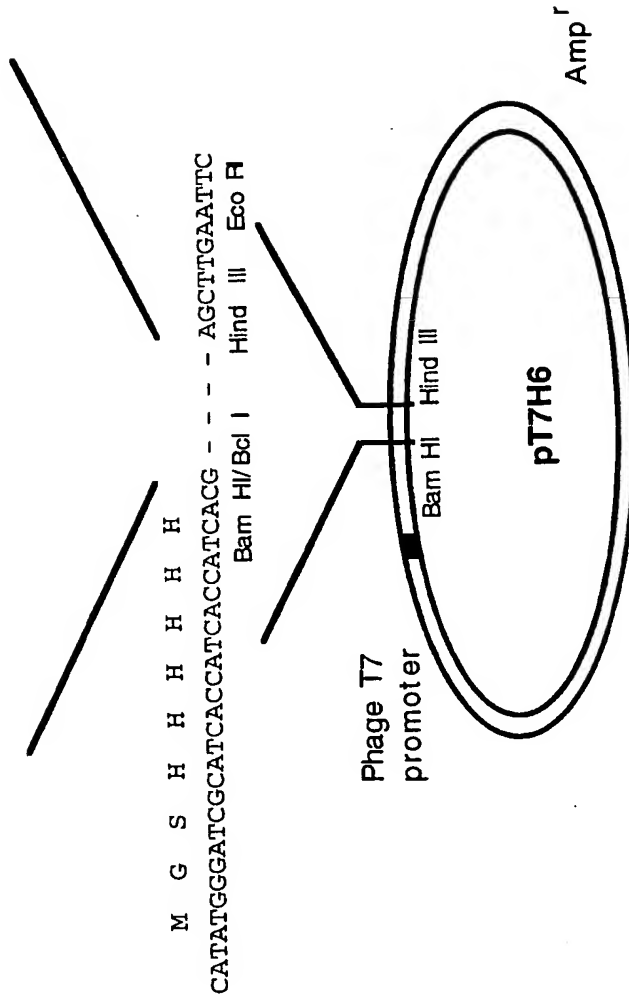


Fig. 3

4/21

H6FXtripa fusion protein

```
1  M G S H H H H H H G S I Q G R S P G T E P P T Q K P K K I V 30
31 N A K K D V V N T K M F E E L K S R L D T L A Q E V A L L K 60
61 E Q Q A L Q T V S L K G S * 73
```

H6FXtripB fusion protein

```
1  M G S H H H H H H G S I Q G R S P G T E P P T Q K P K K I V 30
31 N A K K D V V N T K M F E E L K S R L D T L A Q E V A L L K 60
61 E Q Q A L Q T G S * 69
```

Fig. 4

5/21

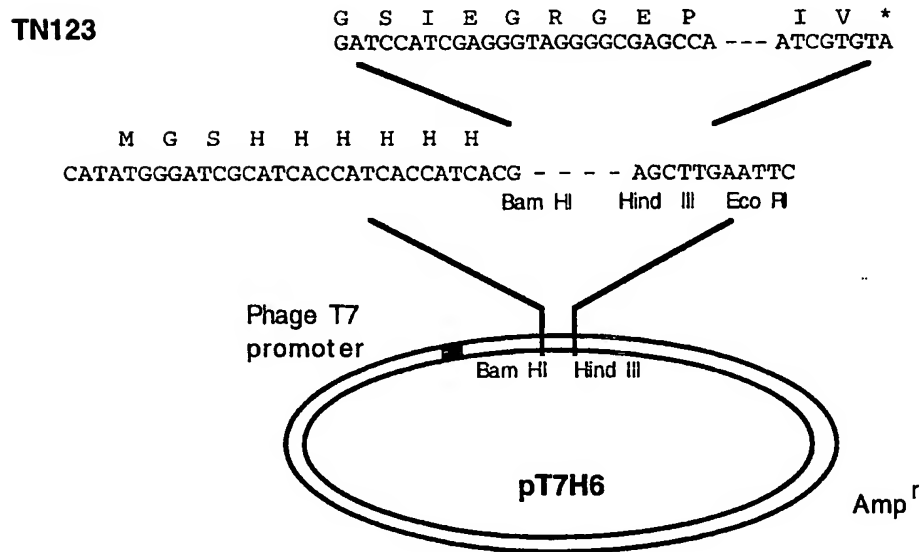
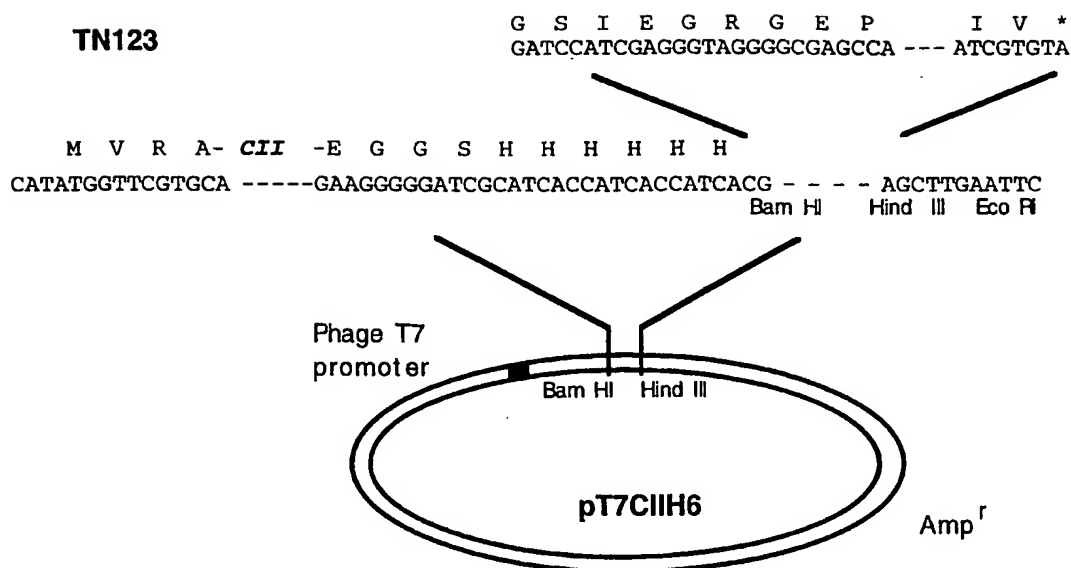


Fig. 5

6/21

CIIH6FXTN123 fusion protein

```
1  MVRANKRNEALRIESALLNKIAMLGTEKTA 30
31  EGGSHHHHHHGSIEGRGEPPTQKPKKIVNA 60
61  KKDVVNTKMFEEELKSRLDTLAQEVALLEKEQ 90
91  QALQTVCLKGTKVHMKCFLAFTQTKTFHEA 120
121 SEDCISRGGTLPSTPQTGSENDALYEYLRQS 150
151 VGNEAEIWLGLNDMAAEGTWVDMTGARIAY 180
181 KNWETEITAQPDGGKTENCAVLSGAANGKW 210
211 FDKRCRDQLPYICQFGIV* 228
```

H6FXTN123 fusion protein

```
1  MGSHHHHHHGSIEGRGEPPTQKPKKIVNAK 30
31  KDVVNTKMFEEELKSRLDTLAQEVALLEKEQQ 60
61  ALQTVCLKGTKVHMKCFLAFTQTKTFHEAS 90
91  EDCISRGGTLPSTPQTGSENDALYEYLRQSV 120
121 GNEAEIWLGLNDMAAEGTWVDMTGARIAYK 150
151 NWETEITAQPDGGKTENCAVLSGAANGKWF 180
181 DKRCRDQLPYICQFGIV* 197
```

Fig. 6

7/21

TN12 G S I E G R G E P P Q T V *
 GATCCATCGAGGGTAGGGGCGAGCCACCA ---- CAGACGGTCTA

TN23 G S I Q G R V V N T G I V *
 GATCCATCCAGGGTAGGGTTGTGAACACA ---- GGGATCGTGTA

TN3 G S I E G R A L Q G I V *
 GATCCATCGAGGGTAGGGCCCTGCAG ---- GGGATCGTGTA

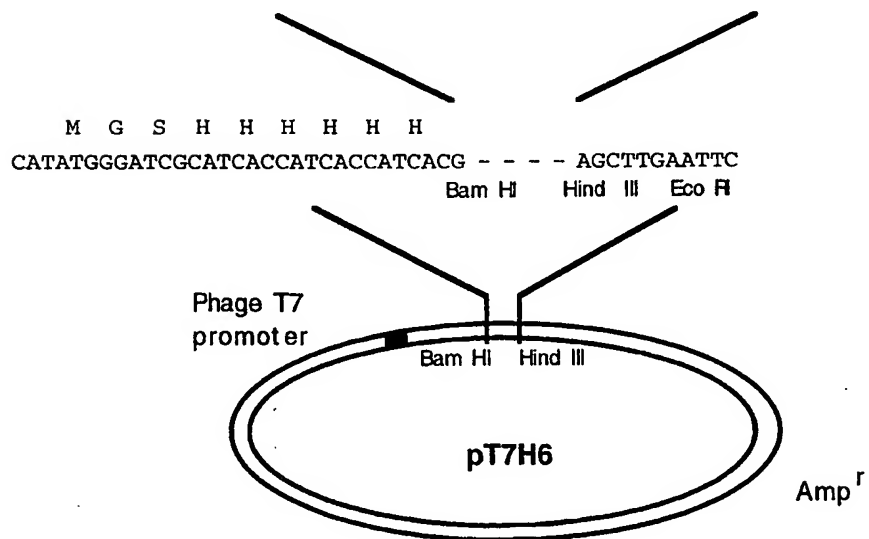


Fig. 7

8/21

H6FXTN12 fusion protein

```
1  M G S H H H H H H G S I E G R G E P P T Q K P K K I V N A K 30
31 K D V V N T K M F E E L K S R L D T L A Q E V A L L K E Q Q 60
61 A L Q T V * 65
```

H6FXTN23 fusion protein

```
1  M G S H H H H H H G S I Q G R V V N T K M F E E L K S R L D 30
31 T L A Q E V A L L K E Q Q A L Q T V C L K G T K V H M K C F 60
61 L A F T Q T K T F H E A S E D C I S R G G T L S T P Q T G S 90
91 E N D A L Y E Y L R Q S V G N E A E I W L G L N D M A A E G 120
121 T W V D M T G A R I A Y K N W E T E I T A Q P D G G K T E N 150
151 C A V L S G A A N G K W F D K R C R D Q L P Y I C Q F G I V 180
181 *
```

H6FXTN3 fusion protein

```
1  M G S H H H H H H G S I E G R A L Q T V C L K G T K V H M K 30
31 C F L A F T Q T K T F H E A S E D C I S R G G T L S T P Q T 60
61 G S E N D A L Y E Y L R Q S V G N E A E I W L G L N D M A A 90
91 E G T W V D M T G A R I A Y K N W E T E I T A Q P D G G K T 120
121 E N C A V L S G A A N G K W F D K R C R D Q L P Y I C Q F G 150
151 I V * 152
```

Fig. 8

9/21

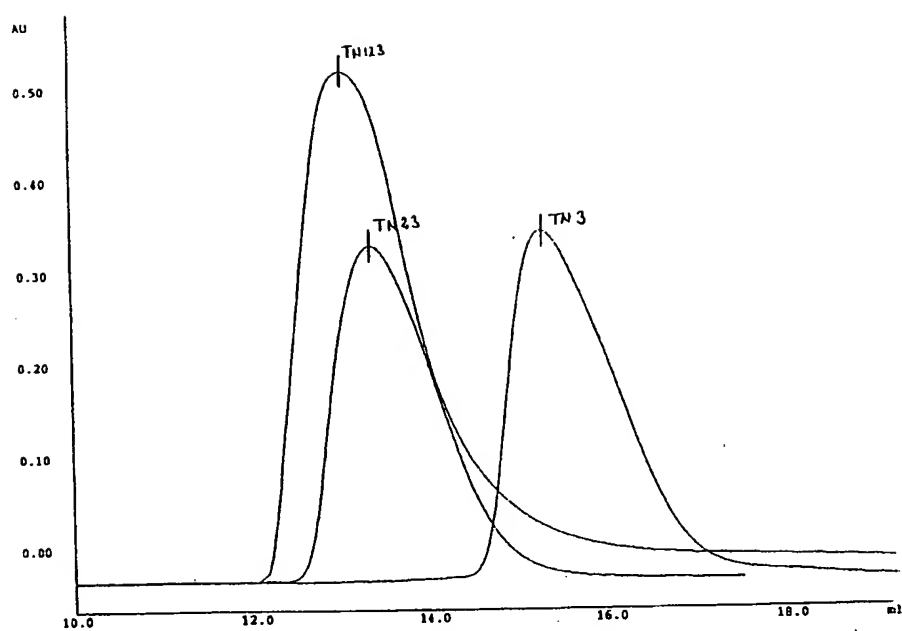


Fig. 9

10/21

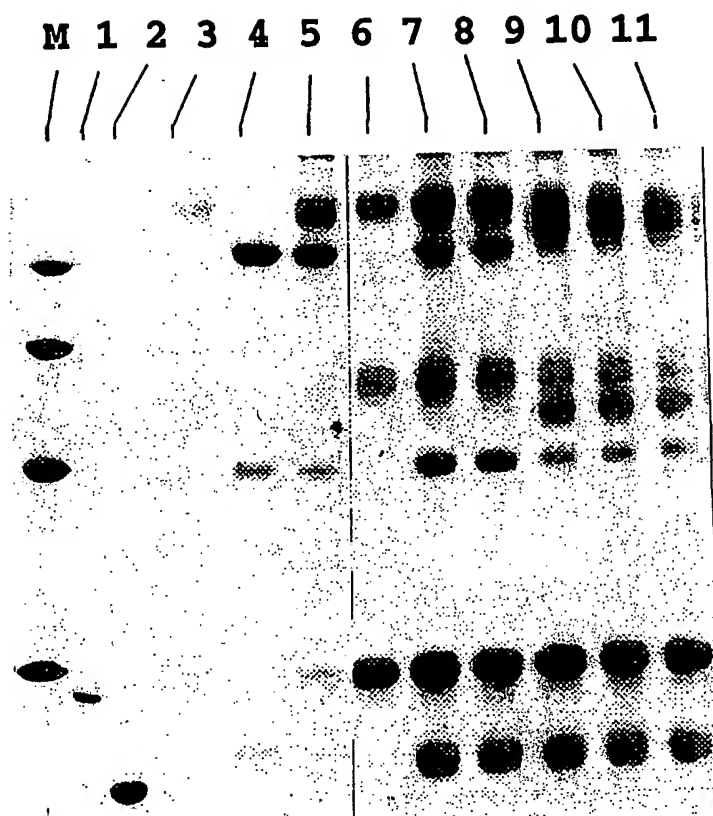


Fig. 10

11/21

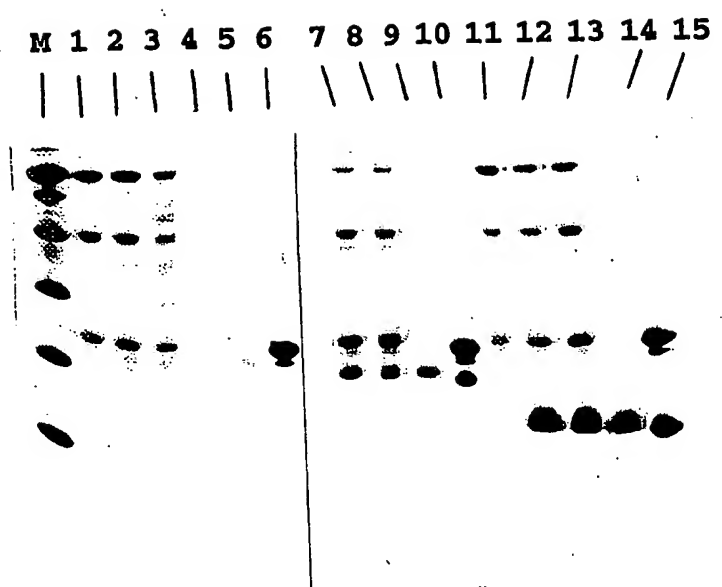


Fig. 11

12/21



Fig. 12

13/21

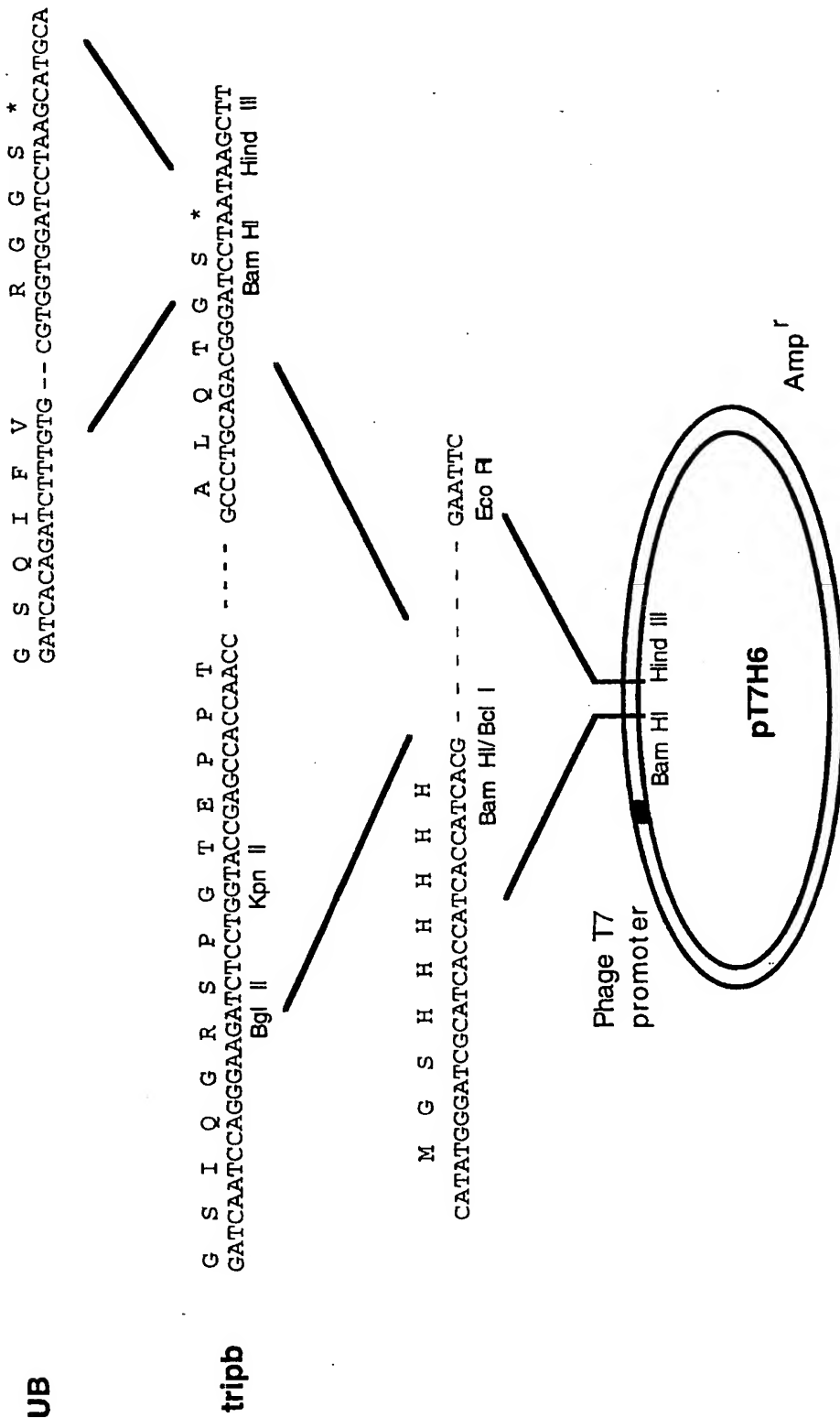


Fig. 13

Fig. 13

14/21

H6FXtripb-UB fusion protein

```
1  M G S H H H H H H G S I Q G R S P G T E P P T Q K P K K I V 30
31 N A K K D V V N T K M F E E L K S R L D T L A Q E V A L L K 60
61 E Q Q A L Q T G S Q I F V K T L T G K T I T L E V E P S D T 90
91 I E N V K A K I Q D K E G I P P D Q Q R L I F A G K Q L E D 120
121 G R T L S D Y N I Q K E S T L H L V L R L R G G S * 145
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Fig. 14

15/21

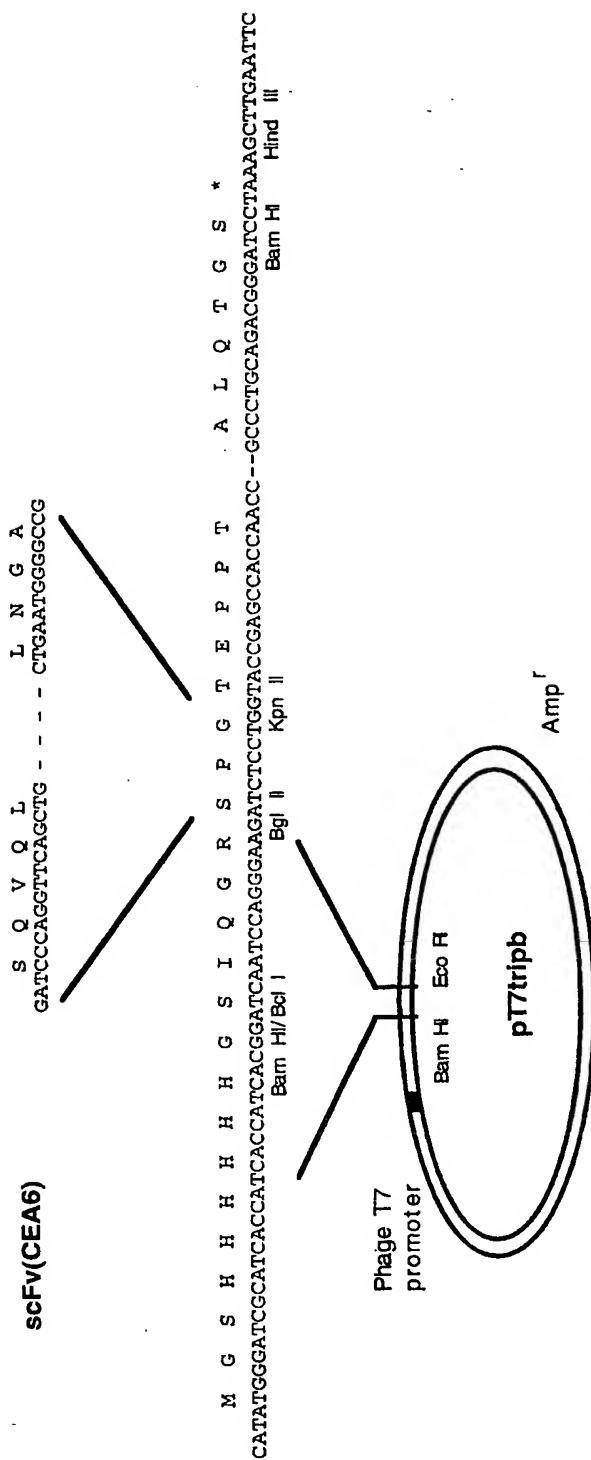


Fig. 15

16/21

H6FXscFv(CEA6)-triplb fusion protein

```
1 M G S H H H H H H G S I Q G R S Q V Q L Q Q S G A E V K K P 30
31 G S S V K V S C K A S G G T F S N S P I N W L R Q A P G Q G 60
61 L E W M G S I I P S F G T A N Y A Q K F Q G R L T I T A D E 90
91 S T S T A Y M E L S S L R S E D T A V Y Y C A G R S H N Y E 120
121 L Y Y Y Y M D V W G Q G T M V T V S S G G G G S G G G G S G 150
151 G G G S D I Q M T Q S P S T L S A S I G D R V T I T C R A S 180
181 E G I Y H W L A W Y Q Q K P G K A P K L L I Y K A S S L A S 210
211 G A P S R F S G S G S G T D F T L T I S S L Q P D D F A T Y 240
241 Y C Q Q Y S N Y P L T F G G G T K L E I K R A A A E Q K L I 270
271 S E E D L N G A G T E P P T Q K P K K I V N A K K D V V N T 300
301 K M F E E L K S R L D T L A Q E V A L L K E Q Q A L Q T G S 330
331 *
```

Fig. 16

17/21

scFv(CEA6) S Q V Q L L N G A
GATCCAGGTTACGCTG - - - CTGAATGGGGCCTA

M G S H H H H H G S I Q G R S P G T E P P T A L Q T G S *
CATATGGGATCGCATCACCATCACCGATCAATCCAGGGAAGATCTCTGGTACCGAGCCACCACC--GCCCTGCAGACGGGATCCTAAAGCTTGAATTC
Bam HI Bcl I Bgl II Kpn II Bam HI Hind III

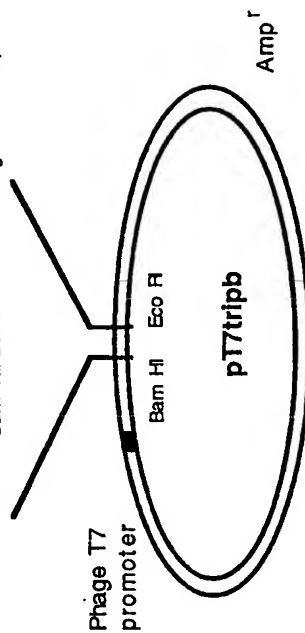


Fig. 17

18/21

H6FXtripb-scFv(CEA6) fusion protein

```
1 M G S H H H H H H G S I Q G R S P G T E P P T Q K P K K I V 30
31 N A K K D V V N T K M F E E L K S R L D T L A Q E V A L L K 60
61 E Q Q A L Q T G S Q V Q L Q Q S G A E V K K P G S S V K V S 90
91 C K A S G G T F S N S P I N W L R Q A P G Q G L E W M G S I 120
121 I P S F G T A N Y A Q K F Q G R L T I T A D E S T S T A Y M 150
151 E L S S L R S E D T A V Y Y C A G R S H N Y E L Y Y Y Y M D 180
181 V W G Q G T M V T V S S G G G G S G G G G S G G G G S D I Q 210
211 M T Q S P S T L S A S I G D R V T I T C R A S E G I Y H W L 240
241 A W Y Q Q K P G K A P K L L I Y K A S S L A S G A P S R F S 270
271 G S G S G T D F T L T I S S L Q P D D F A T Y Y C Q Q Y S N 300
301 Y P L T F G G G T K L E I K R A A A E Q K L I S E E D L N G 330
331 A *
```

Fig. 18

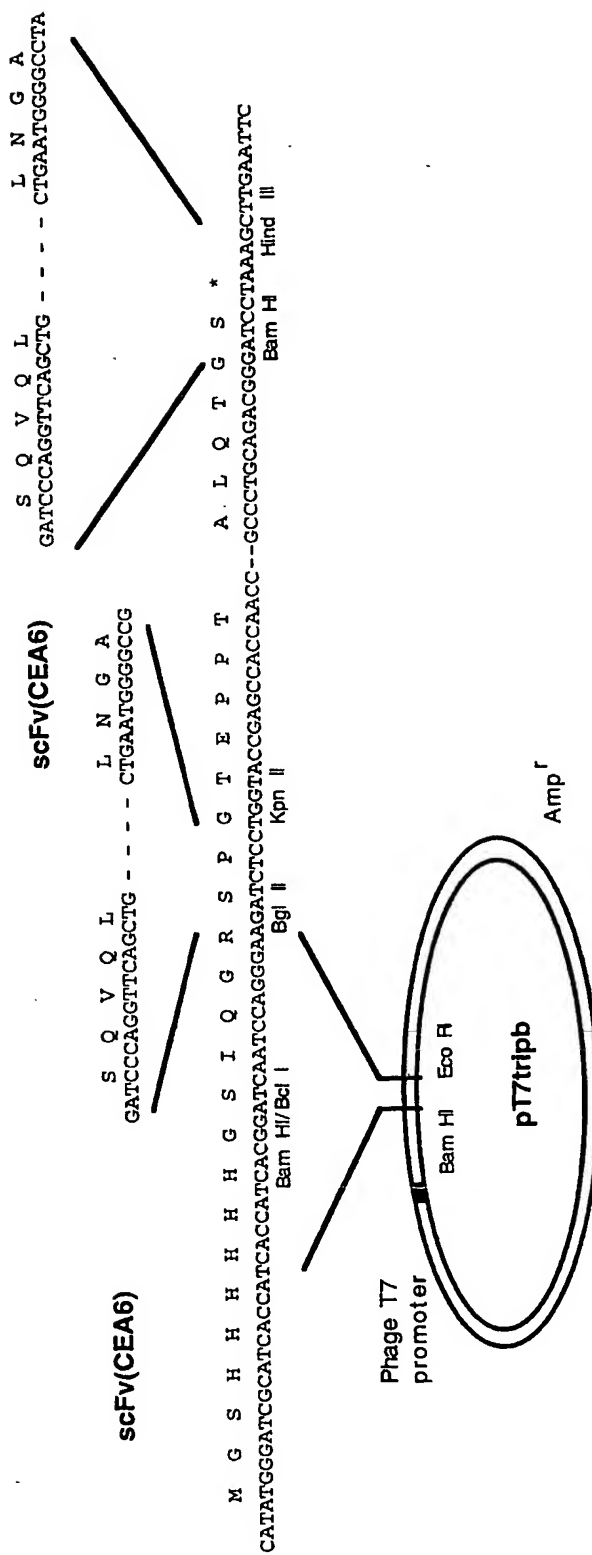


Fig. 19

20/21

H6FXscFv(CEA6)-triph-scFv(CEA6) fusion protein

1 M G S H H H H H H G S I Q G R S Q V Q L Q Q S G A E V K K P 30
31 G S S V K V S C K A S G G T F S N S P I N W L R Q A P G Q G 60
61 L E W M G S I I P S F G T A N Y A Q K F Q G R L T I T A D E 90
91 S T S T A Y M E L S S L R S E D T A V Y Y C A G R S H N Y E 120
121 L Y Y Y Y M D V W G Q G T M V T V S S G G G G S G G G G S G 150
151 G G G S D I Q M T Q S P S T L S A S I G D R V T I T C R A S 180
181 E G I Y H W L A W Y Q Q K P G K A P K L L I Y K A S S L A S 210
211 G A P S R F S G S G S G T D F T L T I S S L Q P D D F A T Y 240
241 Y C Q Q Y S N Y P L T F G G G T K L E I K R A A A E Q K L I 270
271 S E E D L N G A G T E P P T Q K P K K I V N A K K D V V N T 300
301 K M F E E L K S R L D T L A Q E V A L L K E Q Q A L Q T G S 330
331 Q V Q L Q Q S G A E V K K P G S S V K V S C K A S G G T F S 360
361 N S P I N W L R Q A P G Q G L E W M G S I I P S F G T A N Y 390
391 A Q K F Q G R L T I T A D E S T S T A Y M E L S S L R S E D 420
421 T A V Y Y C A G R S H N Y E L Y Y Y Y M D V W G Q G T M V T 450
451 V S S G G G G S G G G G S G G G G S D I Q M T Q S P S T L S 480
481 A S I G D R V T I T C R A S E G I Y H W L A W Y Q Q K P G K 510
511 A P K L L I Y K A S S L A S G A P S R F S G S G S G T D F T 540
541 L T I S S L Q P D D F A T Y Y C Q Q Y S N Y P L T F G G G T 570
571 K L E I K R A A A E Q K L I S E E D L N G A * 592

Fig. 20

21/21

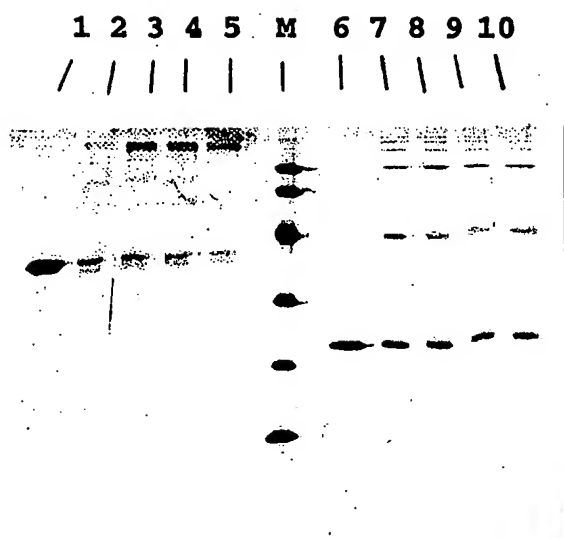


Fig. 21

INTERNATIONAL SEARCH REPORT

International Application No

PCT/DK 98/00245

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/12 C12N15/62 C12N1/21 C07K14/47 C07K16/00
C07K19/00 A61K31/70 A61K48/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 94 18227 A (DENZYME APS ; THOGERSEN HANS CHRISTIAN (DK); HOLTET THOR LAS (DK);) 18 August 1994 cited in the application see page 52, line 1 - line 14; figures 20, 21; example 9	1-55
A	--- L. BERGLUND AND T.E. PETERSEN: "The gene structure of tetranectin, a plasminogen binding protein" FEBS LETTERS, vol. 309, no. 1, 1992, pages 15-19, XP002077572 ELSEVIER, AMSTERDAM, NL cited in the application see the whole document --- -/--	1-55



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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Date of the actual completion of the international search

16 September 1998

Date of mailing of the international search report

01/10/1998

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Authorized officer

Hornig, H

INTERNATIONAL SEARCH REPORT

Int lional Application No

PCT/DK 98/00245

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	EP 0 206 400 A (TNO) 30 December 1986 see the whole document ----	1-55
A	WO 95 31540 A (MEDICAL RES COUNCIL ;HOPPE HANS JUERGEN (GB); REID KENNETH BANNERM) 23 November 1995 cited in the application see the whole document ----	1-55
A	WO 96 37621 A (MORPHOSYS PROTEINOPTIMIERUNG ;PACK PETER (DE); HOESS ADOLF (DE)) 28 November 1996 see the whole document ----	1-55
A	OSBOURN J K ET AL: "GENERATION OF A PANEL OF RELATED HUMAN SCFV ANTIBODIES WITH HIGH AFFINITIES FOR HUMAN CEA" IMMUNOTECHNOLOGY, vol. 2, no. 3, September 1996, pages 181-196, XP000645453 see the whole document ----	1-55
P,A	HOLTET T L ET AL: "Tetranectin, a trimeric plasminogen-binding C-type lectin." PROTEIN SCIENCE, (1997 JUL) 6 (7) 1511-5. JOURNAL CODE: BNW. ISSN: 0961-8368., XP002077573 see the whole document ----	1
P,A	KASTRUP J S ET AL: "Crystal structure of tetranectin, a trimeric plasminogen-binding protein with an alpha-helical coiled coil." FEBS LETTERS, (1997 JUL 28) 412 (2) 388-96. JOURNAL CODE: EUH. ISSN: 0014-5793., XP002077574 see the whole document -----	1

INTERNATIONAL SEARCH REPORT

International application No.

PCT/DK 98/00245

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

see further information sheet
2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Although claims 35, 36, 39, 47-54 and claim 40 as far as in vivo methods are concerned are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

Although claim 55 as far as in vivo methods are concerned is directed to a diagnostic method practised on the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/DK 98/00245

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9418227 A	18-08-1994	AU 674568 B	02-01-1997
		AU 6038094 A	29-08-1994
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